Wolf D. Kuhlmann

Immuno Enzyme Techniques in Cytochemistry

Wolf D. Kuhlmann

Immuno Enzyme Techniques in Cytochemistry

verlag chemie
Weinheim
Deerfield Beach, Florida
Basel

Dr. Wolf D. Kuhlmann, M.D.
Laboratory of Experimental Medicine
and Immunocytochemistry
Deutsches Krebsforschungszentrum
Im Neuenheimer Feld 280
D-6900 Heidelberg
Federal Republic of Germany

Heisenberg Research Fellow,
Deutsche Forschungsgemeinschaft;
formerly Research Fellow,
Institut de Recherches Scientifiques
sur le Cancer, Villejuif, France

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

To the memory of

Wilhelm Bernhard

(1920 – 1978)

to whom I owe so much

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. Kuhl-

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
Brown University
Providence, Rhode Island
U.S.A.

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

Contents

	Introduction	1
	References	3
1	**Basic Methods and Aspects in Immunological Reagent Preparation**	5
1.1	Evaluation of Antigens and Antibodies	5
1.1.1	Gel electrophoresis	6
1.1.2	Immunoelectrophoretic analysis and double diffusion in gels	9
1.1.3	Radial immunodiffusion	11
1.1.4	Electroimmunodiffusion	12
1.2	Purification of Antigens	13
1.2.1	Ion exchange chromatography	17
1.2.2	Gel filtration technique	19
1.2.3	Chromatofocusing	23
1.2.4	Preparative polyacrylamide gel electrophoresis	26
1.2.5	Affinity chromatography	28
1.3	Preparation of Immune Sera and Antibodies	31
1.3.1	Lymphoid cells and antibody production	31
1.3.2	Hyperimmunization of animals: polyclonal and monoclonal antibodies	32
1.3.3	Isolation of antibodies by affinity chromatography	36
	References	38
2	**Preparation of Immunohistological Reagents**	43
2.1	Marker Molecules in Light and Electron Microscopy	43
2.2	Coupling Reagents and Conjugation Procedures	46
2.2.1	"One-stage" and "two-stage" reactions	47
2.2.2	Antibody-enzyme conjugation by use of glutaraldehyde	48
2.3	Purification and Characterization of Enzyme Conjugates	50
2.3.1	Purification by gel filtration	50
2.3.2	Gel filtration and lectin binding technique	50
2.3.3	Qualitative and quantitative characterization of conjugates	51

2.4	Production of Soluble Peroxidase-Antiperoxidase Complexes	55
	References	57
3	**Histological Immuno Enzyme Techniques**	61
3.1	Direct and Indirect Immunolocalizations	61
3.2	Enzyme Cytochemistry Procedures	63
3.3	Control of Specificity	66
3.4	Microscopy, Counterstaining and Microphotography	67
	References	69
4	**General Preparation of Biological Specimens**	71
4.1	Tissue Fixation	72
4.2	Tissue Dehydration	75
4.3	Tissue Embedment	76
4.4	Antigen Protective Measures	79
	References	83
5	**Immunoperoxidase Histology in the Light Microscope**	85
5.1	General Methods of Antigen Detection in Paraffin Sections	85
5.2	Immunoperoxidase Localization of Cell-associated Marker Molecules (Mucus Neutral Glycoproteins) in Gastrointestinal Mucosa	87
5.3	Antigen Detection in Semithin Resin Sections	96
5.4	Immunolocalization of Antigens in Monolayer Cultures	101
	References	103
6	**Immunohistological Assays for Electron Microscopy**	107
6.1	Immuno-staining in Preembedment Period	108
6.2	Immuno-staining in Postembedment Period	116
6.3	Immuno-staining in Cryo-ultramicrotomy	121
6.4	Miscellaneous Techniques	125
	References	125
7	**Immunohistological Specificity**	131
7.1	False Positive Staining	131
7.2	False Negative Staining	135
	References	136
8	**Practical Aspects**	137
8.1	Polyacrylamide Gel Electrophoresis	137
8.2	Immunological Gel Diffusion	139
8.3	Immunological Precipitation of Antigen and Antibody	141
8.4	Chromatography and Gel Filtration	142
8.5	Spectrophotometrical Methods	143

8.6 Histological Preparations 146
References 154

Index 157

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.

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.

References

1. A. G. E. Pearse: *Histochemistry. Theoretical and Applied.* Churchill-Livingstone, Edinburgh 1972 and London 1980.
2. H. Lis and N. Sharon, "The biochemistry of plant lectins (phytohemagglutinins)", *Ann. Rev. Biochem.* **42** (1973) 541.
3. S. H. Barondes, "Lectins: their multiple endogenous cellular functions", *Ann. Rev. Biochem.* **50** (1981) 207.
4. A. H. Coons, H. J. Creech and R. N. Jones, "Immunological properties of an antibody containing a fluorescent group", *Proc. Soc. Exp. Biol.* **47** (1941) 200.
5. A. H. Coons and M. Kaplan, "Localization of antigen in tissues: improvement in a method for the detection of antigen by means of fluorescent antibody", *J. Exp. Med.* **91** (1950) 1.
6. R. C. Nairn: *Fluorescent Protein Tracing.* Churchill-Livingstone, Edinburgh 1976.
7. J. Almeida, B. Cinader and A. Howartson, "The structure of antibody-antigen complexes. A study by electron microscopy", *J. Exp. Med.* **118** (1963) 327.
8. J. K. Blasie and M. M. Dewey, "Electron microscope and low angle x-ray diffraction studies on outer segment membranes from the retina of the frog", *J. Mol. Biol.* **14** (1965) 143.
9. S. J. Singer, "Preparation of an electron-dense antibody conjugate", *Nature (Lond.)* **183** (1959) 1523.
10. W. D. Kuhlmann, "Ultrastructural immunoperoxidase cytochemistry", *Prog. Histochem. Cytochem.* **10/1** (1977) 1.
11. L. A. Sternberger: *Immunocytochemistry.* John Wiley & Sons, New York 1979.

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]

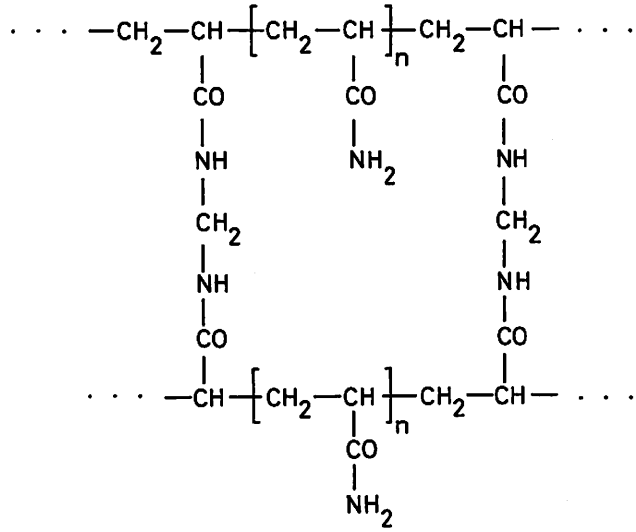
[a)] Sensitivity ca. 10^{-5} g/mL; in combination with enzyme labeled antibodies/antigens and by revealing the enzyme activity, sensitivity will give values of ca. 10^{-7} g/mL;
[b)] Not principle usage;

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.

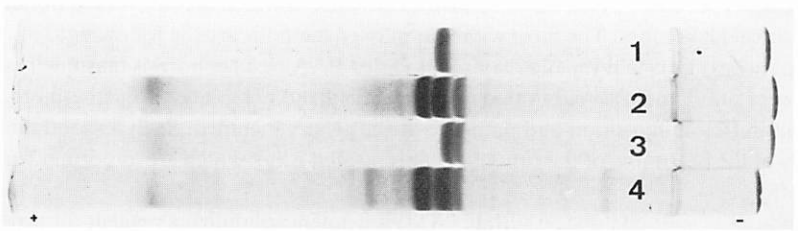


Figure 1-1. PAA electrophoresis; isolated mouse AFP (1), mouse amniotic fluid (2), isolated rat AFP (3), rat amniotic fluid (4).

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.

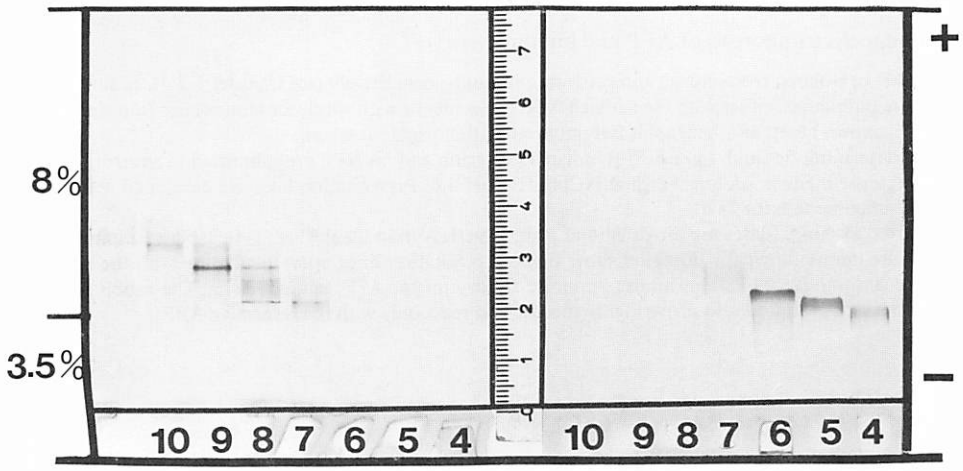


Figure 1-2. Analytical PAA electrophoresis of fractions from preparative PAA electrophoresis; fraction numbers (4 – 10) correspond to eluted gel strips; Coomassie blue (left) and Alcian blue (right) stainings, respectively.

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).

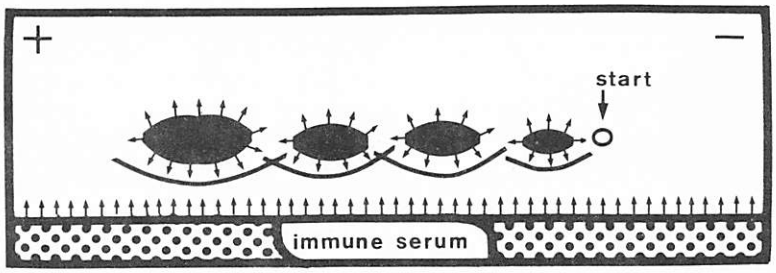


Figure 1-3. Immunoelectrophoresis, schematically.

Precipitating antibodies will form, by reaction with corresponding antigens, curved precipitation bands. Thus, antigens can be characterized by their electrophoretic mobility and their specific immunological reaction. Furthermore, chemical *in situ* characterization can be added such as detection of enzyme activities. The following example shows the immunoelectrophoretic analysis of isolated mouse and rat AFP molecules and their respective anti-AFP immune sera prepared by hyperimmunization of rabbits.

Immunoelectrophoresis of AFP and immune sera:

1. AFP ist isolated from mouse and rat amniotic fluids, respectively (see Chapter 1.2.5). Individual rabbits are immunized either with the isolated AFP molecules or with whole amniotic fluids (mouse amniotic fluid is shown here); another rabbit is immunized with normal rat serum.
2. Mouse amniotic fluid, mouse AFP, normal rat serum and rat AFP are submitted to electrophoresis in 1 % agarose made in sodium Veronal-HCl buffer pH 8.6. Precipitation lines are developed with the different immune sera for 24 h.
3. After washing, plates are air-dried and stained with Amido black (Fig. 1-4). Isolated mouse and rat AFP are immunologically pure and show single precipitation lines upon incubation with the respective rabbit anti-mouse AFP, anti-mouse amniotic fluid, anti-rat AFP immune sera. The rabbit anti-AFP immune sera produced also prove highly specific and react only with the respective AFPs.

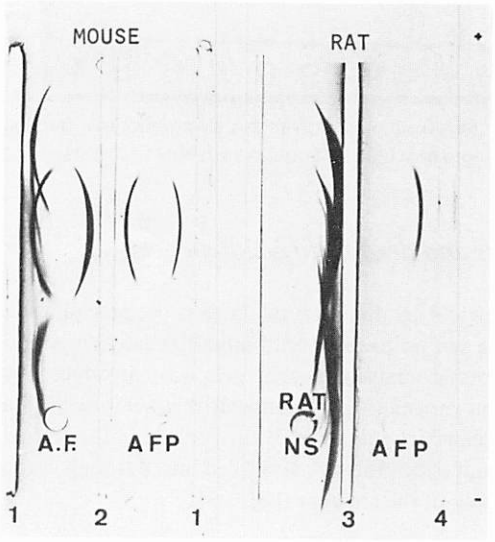


Figure 1-4. Immunoelectrophoretic analysis; mouse amniotic fluid (A.F.), normal rat serum (RAT NS), anti-mouse amniotic fluid (1), anti-mouse AFP (2), anti-rat normal serum (3) and anti-rat AFP (4).

For the method of double diffusion in gel, agar or agarose gels are cast in a similar manner on glass plates. Then, wells are cut in the gels and filled with antigen and antibody solutions [15]. Without previous electrophoresis but simply by radial diffusion of both solutions, antigens and antibodies will react at a given equivalence so that antibodies will precipitate the corresponding antigens. The precipitation lines obtained allow immunological estimation of either of the reactants (Fig. 1-5).

A certain antigen under study will be prepared at different times and under different conditions within the years of experimentation. Double diffusion in gel is a suitable method to control immunological homogeneity between previously obtained standard antigen and

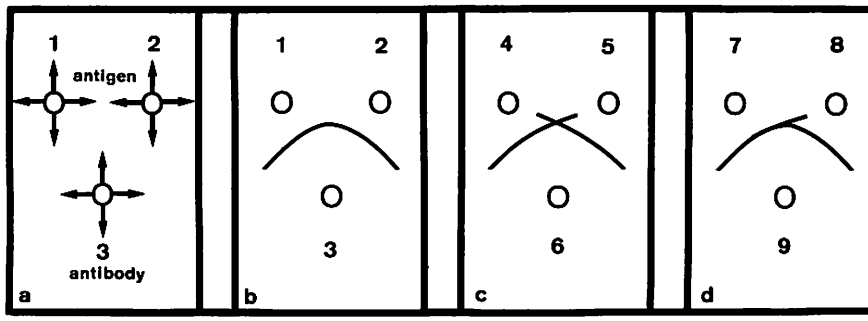


Figure 1-5. Double diffusion in gel, schematically; (a) antigens and antibodies diffuse from their wells into the gel; (b) when the same antigen is placed in wells 1 and 2, presence of identity is seen by continuous precipitation line (antibody in well 3); (c) two different antigens in wells 4 and 5 react with immune serum (well 6) against each of the antigens and show non-identity by crossed precipitation; (d) spur formation due to partial identity is observed when an antigen (well 7) and its antiserum (well 9) precipitate in presence of a cross-reacting antigen (well 8).

immune sera and some newer preparations. For such examination, antigens and immune sera/antibodies may be cross-matched. A simplified example is shown in Fig. 1-6.

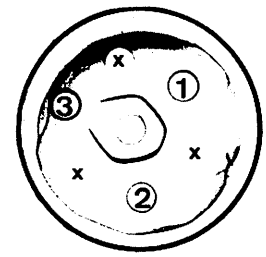


Figure 1-6. Ouchterlony's double diffusion in gel technique; central well is filled with rabbit anti-rat amniotic fluid immune serum; several AFP preparations (1, 2, 3) and a standard AFP (x) are placed around.

Double diffusion in gel for AFP screening:

1. Rat AFP preparations from several different runs on affinity chromatography columns (Chapter 1.2.5) are tested against a defined AFP standard in the double diffusion in gel technique.
2. A central well is punched into the gel and filled with a rabbit anti-rat amniotic fluid immune serum. Further wells are cut around this central well and filled with the unknown probes and the positive control in an alternative manner.
3. After overnight incubation, plates are washed, dried and stained with Amido black (Fig. 1-6). Two of the examined preparations contain AFP; the third one is negative. Other precipitation lines are not detected. Under these simplified conditions, the continous precipitation reaction is favorable for homogeneity between the standard and two of the tested AFP preparations. Further controls with other immune sera and by use of a panel of different antigen preparations can be included.

1.1.3 Radial immunodiffusion

Radial immunodiffusion according to Mancini et al. [16] is widely employed for quantitative measurements of either antigens or antibodies. Agar or agarose gels are cast on exact

horizontally arranged glass plates as described above with the exception that antigen (when the antibody is quantitated) or specific immune serum (when the antigen is quantitated) are uniformly incorporated into the gel. Wells are cut in the gel layer and filled with exact volumes of appropriate dilutions of the probe. Parallel to this, wells are filled with dilutions from a standard of known concentration. By diffusion and subsequent immunoreaction, rings of precipitation are formed. The areas of immunoprecipitate are proportional to the amount of antigen or antibody, respectively. The square of the radius is determined which is directly proportional to the probe concentration. Results from the known standards provide the calibration curves.

Mancini's method is easily installed for measurements of precipitating antibodies in immune sera, γ-globulin fractions etc. or for quantitation of desired antigen e.g. in tissue extracts, in body fluids and, of course, in the course of antigen purification. In the following, an example is given for AFP quantitation in tissue extracts.

Radial immunodiffusion for AFP quantitation:

1. Extracts are prepared from AFP producing hepatoma livers in order to quantitate their AFP content; an isolated AFP serves as standard.
2. A series of wells are cut into the anti-AFP containing gel plate and filled with defined volumes of appropriate dilutions of the standard. Another series of wells is used for dilutions of the liver extracts. Diffusion is allowed to proceed for 48 h at room temperature in a humidified chamber.
3. After washing and air-drying, the plates are stained with Amido black; immunoprecipitates are measured (Fig. 1-7). The AFP concentration can be calculated from the established standard curve.

1.1.4 Electroimmunodiffusion

As an alternative to quantitative radial immunodiffusion, Laurell [18] described a procedure by which antigens can be easily and rapidly quantitated. In this technique agar or agarose plates are prepared in a comparable manner inasmuch as an appropriate dilution (determined by trial) of a specific immune serum is uniformly incorporated into the gel. Then, probes are filled in previously cut wells (exact volumes) and submitted to electrophoresis. The antigen migrates into such gel preparations and the specific antigen-antibody precipitate forms a peak (rocket), the height of which is proportional to the antigen concentration.

The sensitivity of the method can be significantly enhanced when the "rockets" are visualized by subsequent incubation with a Sandwich antibody being conjugated with an enzyme. In such enzyme-electroimmunodiffusion, the primary immune serum is incorporated in the gel in much higher dilution. Also, the probes are used in higher dilutions, i.e. the probes can contain much less antigen than in the usual Laurell's technique. After electrophoresis, immunoprecipitates cannot be detected by conventional Amido black staining; rockets only become developed by the amplifying effect of enzyme staining which is performed after subsequent incubation of the gel plates with an enzyme labeled Sandwich antibody. For this purpose, peroxidase labeled antibodies [42] and glucose oxidase labeled antibodies [43] were used.

Quantitation of AFP by electroimmunodiffusion:

1. Extracts are prepared from hepatoma livers and taken for AFP quantitation; an isolated AFP is used as standard.

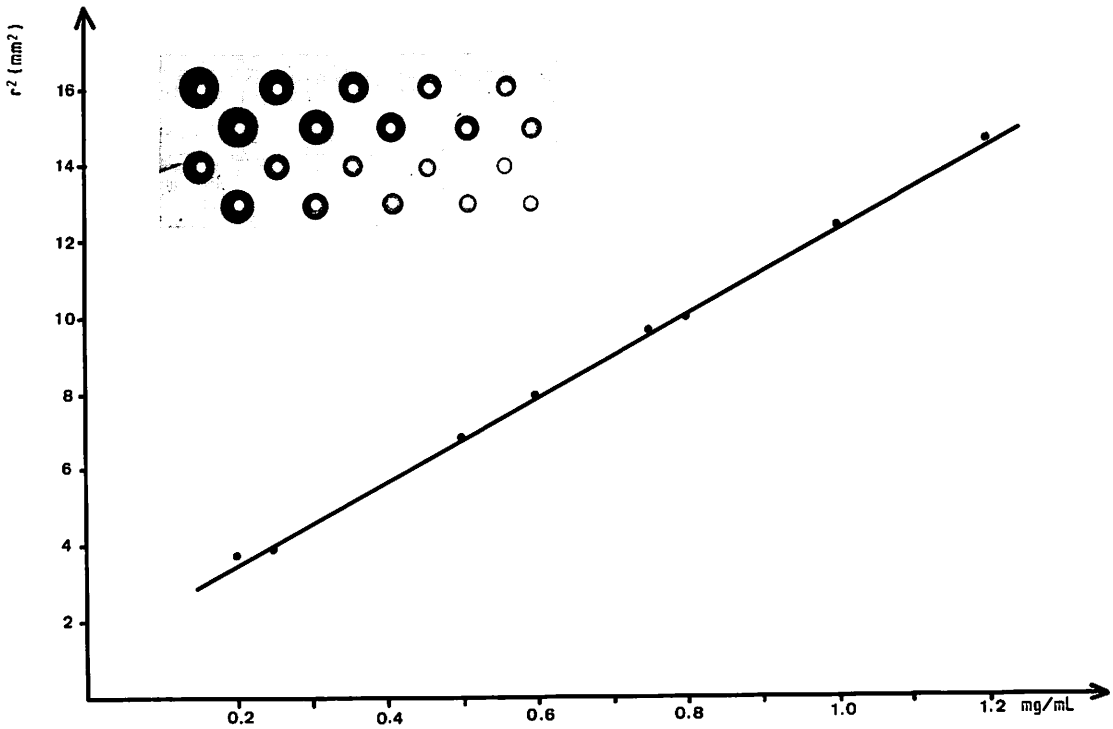


Figure 1-7. Radial immunodiffusion; calibration curve from dilutions of AFP standard. Inset: part of an Amido black stained gel plate.

2. A series of wells are cut into the anti-AFP immune serum containing agarose gel and filled with defined volumes of serial dilutions of standard AFP and defined volumes of dilutions of liver extracts.
3. After electrophoresis for 4 hours, plates are washed overnight, air-dried and stained with Amido black (Fig. 1-8). The heights of the rockets are measured and AFP contents can be calculated by use of the standards plotted in a curve.

As already stated the proposed procedures for antigen and antibody evaluation are only a selection with the advantage that users do not need expensive equipment nor do they need detailed knowledge of immunochemical methodology. For more details (which may be desirable in any case), further reading in respective handbooks of experimental immunochemistry is suggested (ref. in Chapter 1.1).

1.2 Purification of Antigens

It is readily understood that sufficient specificity in the immunostaining of antigen can only be achieved with the use of specific antibody against the given molecule. In case of unavailability of the respective antibody, this must be prepared by appropriate immunization of an animal. Under all circumstances, the antigen used must be a preparation of the highest

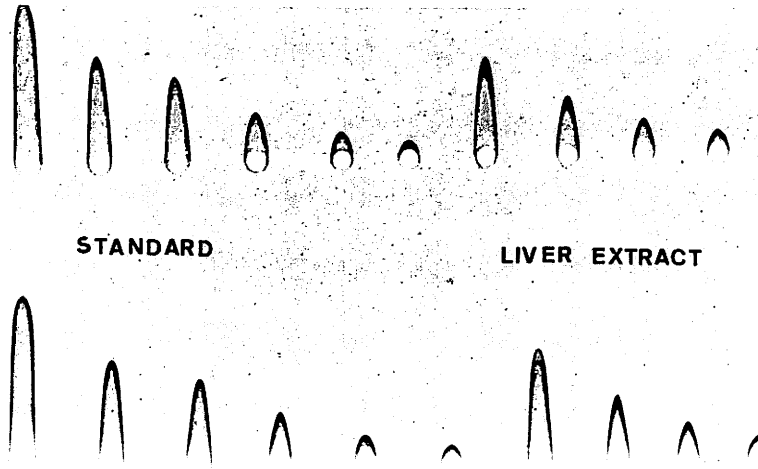


Figure 1-8. Electroimmunodiffusion; see text.

purity because minor contaminants in the immunizing mixture may elicit considerable amounts of unwanted antibodies. In the case of organ-associated antigens, the desired substance may occur only as a minor part in the bulk of all soluble and insoluble components, and its purification will not be achieved in a single step. Usually, a sequence of different procedures must be employed and this holds especially true when one has to begin with crude organ extracts. Principally, a variety of physico-chemical methods are available. Depending on the nature of the molecules under study only certain approaches will yield the necessary degree of purification. It is not a priori defined what procedure will be successful and extensive trials are sometimes necessary. Typical methods applied for the concentration and isolation of proteins from organ extracts are:

- precipitation reactions, e.g. with organic solvents, salt, perchlorid acid;
- chromatography, e.g. ion exchange chromatography, gel filtration, ligand specific affinity chromatography;
- centrifugation, ultracentrifugation, e.g. isopycnic and zonal centrifugation;
- electrophoretic separation, e.g. polyacrylamide or agarose gel as supporting matrix, free electrophoresis.

All the proposed steps must be acompanied by appropriate analysis for which immunological techniques (some which are widely employed have been presented in Chapter 1) prove very valuable. For a complete review of all possible purification techniques and analytical evaluations we refer to respective monographs and handbooks of biochemistry and immunochemistry [7–9, 44]. It is felt, however, that theoretical and practical concepts for the preparation of pure protein antigens should be known to immunohistologists.

In our laboratory, the isolation of gastrointestinal glycoproteins is of primary interest because we want to produce specific antibodies against those molecules and to use them in immunohistological studies of normal and diseased organs of the gastrointestinal tract. The value of gastrointestinal glycoproteins as functional markers in histogenesis and histodifferentiation should be elucidated. Target organs are the liver and gastric and intestinal

mucosae. The oncofetal antigen alpha$_1$-fetoprotein (AFP) was chosen for studies on the liver. In the case of gastrointestinal mucosae, our special interest is focused on neutral glycoconjugates. While the isolation of molecules like AFP is sometimes readily accomplished, purification of other ones like the gastrointestinal glycoconjugates may often be a difficult task. Hence, it is reasonable to look at typical pathways for the isolation of biological macromolecules which will be especially usefull for those who hitherto have not been involved in the preparation of pure antigens. For this purpose, the major steps and peculiarities in the purification of human gastric mucosa glycoconjugates are considered.

Gastrointestinal glycoproteins occur in a variety of mucosal cells and in extracellular mucins. These often seem by histochemical means to be quite homogenous, e.g. as Alcian Blue (acid glycoproteins) or PAS (periodic-acid-Schiff) positive (neutral glycoproteins) substances. However, such molecules exhibit by biochemical and immunochemical means great heterogeneity. In order to separate them into unitary components, methods like ion exchange, molecular size sieving and chromatofocusing were tried. These proved to be useful for successive concentration of the desired molecules, but none of the methods yielded a sufficient degree of purity. With the use of preparative polyacrylamide (PAA) gel electrophoresis, however, added as a final step, we succeeded in the isolation of sufficient amounts of molecules for their analytical characterization and for subsequent immunization of rabbits. The physical nature of high molecular weight glycoproteins often makes their isolation and characterization difficult which is due to the fact that they can form "visco-elastic networks" [45] at higher concentrations. Because of this, small quantities of contaminants are difficult to detect, and clear-cut electrophoretic zones are not always formed.

A flow chart for the preparative isolation of PAS positive neutral glycoproteins from human gastric mucosa and pertinent controls are shown in Fig. 1-9. For a better understanding, the isolation process is briefly summarized.

Mucosa from defined normal human stomach (of known blood group) was dissected free from serosa and muscularis, homogenized (Ultraturrax, max. speed) in cold PBS for 5 min, centrifuged at 10000 g for 20 min and filtered through teflon wool. The precipitate was homogenized and centrifuged twice as above. Supernatants were pooled, extracted with 0.6 mol/L perchloric acid (0 °C) for 20 min, and precipitates were removed by centrifugation. The supernatant was neutralized with 1 mol/L NaOH, dialyzed against PBS and concentrated to 5 mg/mL protein by ultrafiltration. This crude extract from gastric mucosa was still a complex mixture as seen in the first screening and served as starting material for subsequent and stepwise purification: DEAE ion exchange chromatography, gel filtration, chromatofocusing and preparative PAA electrophoresis, sequentially.

Because neutral glycoproteins should be isolated, the various purification steps were monitored in this way: (a) probes from individual fractions of the respective column chromatographs were controlled by a colorimetric assay for glycoproteins which is based on the periodic-acid-Schiff (PAS) reaction [46]; (b) individual fractions from each gradient step / peak of the respective chromatographic runs were pooled and analyzed by zone electrophoresis in PAA with Coomassie Blue (for proteins) and PAS stainings (for neutral glycoproteins), respectively. Selected peaks were submitted to further isolation until the last purification step was reached which was in our case preparative electrophoresis in PAA. Then, individual gel strips were eluted and analyzed.

Useful data on the characteristics of the investigated molecules were then obtained with progress of their purification (e.g. electrophoretic behavior, stainability, molecular weight,

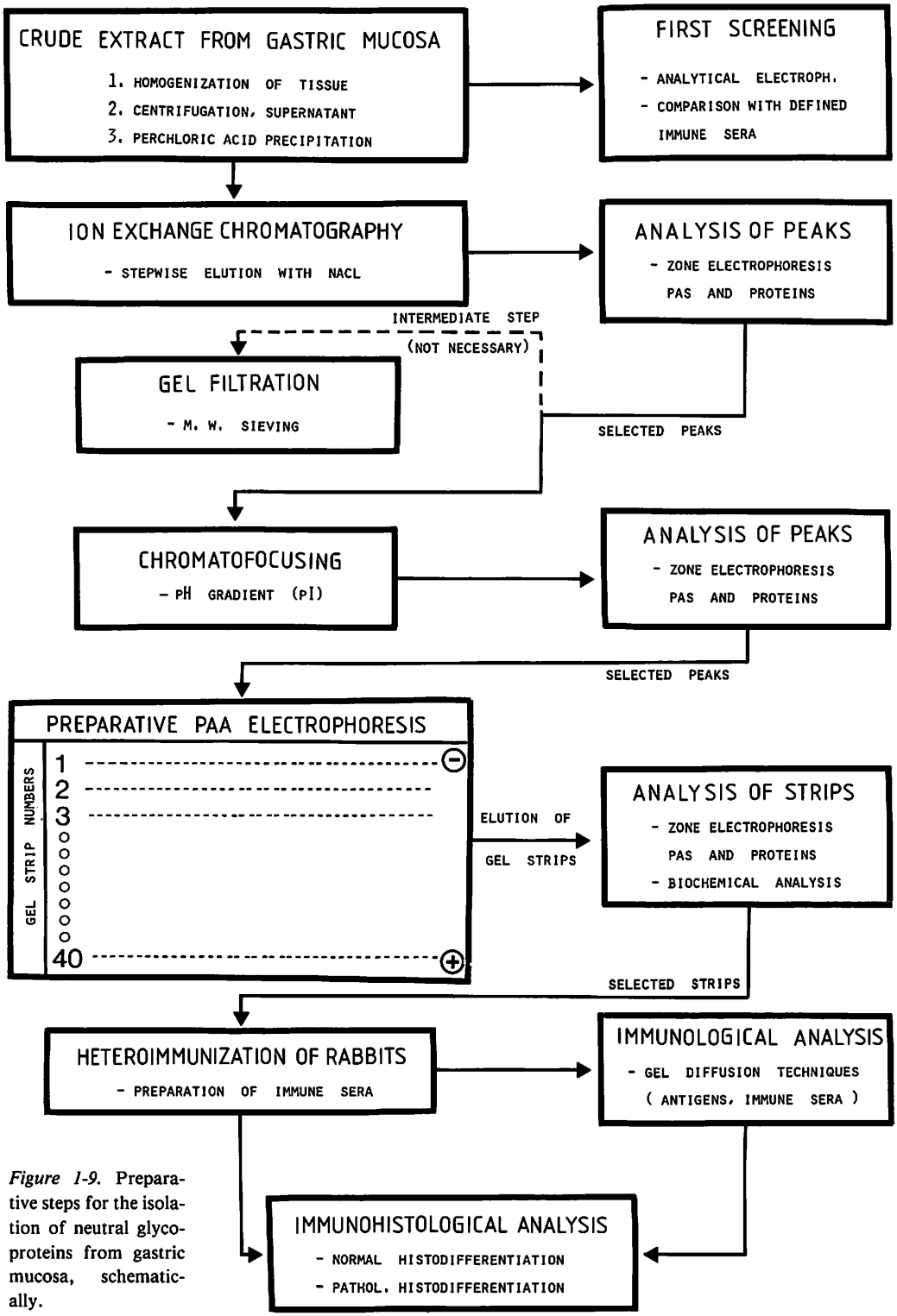


Figure 1-9. Preparative steps for the isolation of neutral glycoproteins from gastric mucosa, schematically.

isoelectric point, immunological parameters). Some more details were further described by biochemical analyses (other than the above ones) such as determination of protein and sugar composition (amino acid analysis; spectrophotometric assays for sugars; gas liquid chromatography for neutral sugars, sialic acids), analytical affinity chromatography (selective immunological and lectin binding studies), just to mention a few. The latter are not the subject of this monograph and will not be presented here in detail.

Purified glycoconjugates were used for the immunization of rabbits. Specificity of the obtained immune sera was controlled by crossmatching with isolated homologous antigens, crude fractions and heterologous antigens in gel diffusion techniques. Hence, purity of a given isolated glycoprotein was further proved by immunological criteria. Since blood group substances are known to occur in mucosa cells, the purity and homogeneity of purified biomolecules (obtained from the stomach of a defined blood group donor) with respect to such substances must be controlled by immunological criteria (specific precipitation reaction, inhibition of hemagglutination). Finally, the immunohistological staining pattern of the produced immune sera against defined glycoconjugates in controlled absorption, non-absorption and blocking tests served as criteria, too. After all these examinations, immunohistological assays with normal and diseased organs could be performed (for application see Chapter 5.2).

Experimental procedures (which can be adapted to other organ antigens) are here described for the purification of gastric glycoconjugates. Because specific immune sera were not available at the beginning of our experiments, affinity chromatography by immunoadsorption was not included in the isolation schedules. An example for the usefulness of affinity chromatography will be given with AFP in Chapter 1.2.5

1.2.1 Ion exchange chromatography

Ion exchange chromatography was introduced by Peterson and Sober [47] and has proved useful for the separation of biological macromolecules. The physical principle of this technique is the attraction of differently charged groups and separation is achieved according to differences in the net charge. Proteins change their net charge as a function of the environmental pH, whereby negatively charged groups can be supplied from carboxylic acid residues (aspartic acid, glutamic acid) at an alkaline pH (uncharged at an acidic pH) and positive groups along the protein molecules are mainly supplied by amino acids like arginine, histidine and lysine at an acidic pH (uncharged at an alkaline pH). In conjugated proteins, e.g. glycoproteins, the conjugated moiety will contain charged groups, too. Fig. 1-10 shows the relation between pH and net charge of an arbitrary protein; for example, at pH 4.5 the net charge is +2 and at pH 9.5 the net charge is -2. Then, the net charge of that protein is zero at a specific pH, e.g. pH 6.5 which is called the isoelectric point (pI) of the protein.

In practice, cellulose based or cross-linked dextran based ion exchangers are used which are substituted by carboxymethyl groups ($-OCH_2COO^{\ominus}$; CM cation exchanger) or diethyl-aminoethyl groups ($-OCH_2CH_2NH^{\oplus}$ $(CH_2CH_3)_2$; DEAE anion exchanger) and, thus, determine the functional characteristics of the adsorbent. Non-ionic interactions between hydroxyl groups of the matrix and polar residues of the probes can be diminished by the addition of urea to the elution buffer [48]. The choice of an ion exchanger depends on the net charge of the molecules to be separated (review at Peterson and Sober [49]).

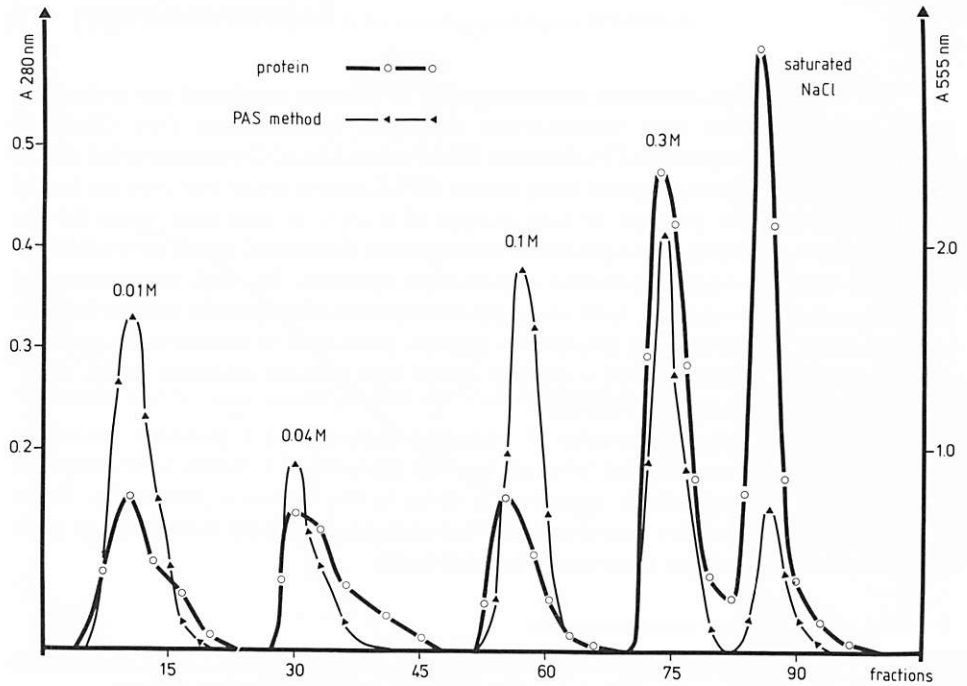


Figure 1-12. DEAE ion exchange chromatography of crude extract from gastric mucosa; elution profile measured for protein content and PAS staining substances.

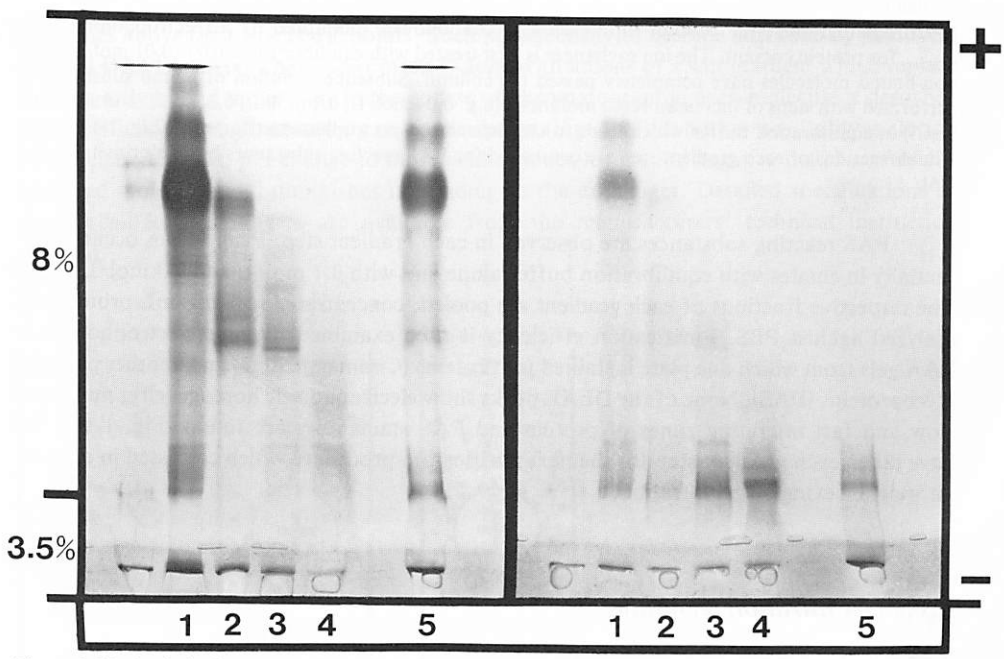


Figure 1-13. Analytical PAA electrophoresis of crude extract from gastric mucosa (1) and after its fractionation by DEAE ion exchange chromatography (2 = 0.01 mol/L; 3 = 0.04 mol/L; 4 = 0.1 mol/L; 5 = 0.3 mol/L); Coomassie blue (left) and PAS (right) stainings, respectively.

gels. The latter are prepared from dextran, acrylamide, agarose or combinations of all of them. Different degrees of concentration or cross-linking enable various fractionation ranges for globular proteins and polysaccharides, and chromatographic resolution is influenced by different particle size grades. Gel filtration is now a routine technique because of its simplicity, reliability and efficiency as a separation method. Several inert media with controlled pore size are commercially available. A selection of gels for the separation of high molecular weight substances is shown in Table 1-2.

The most important applications of gel filtration techniques are (a) separation and isolation of native biomolecules; (b) molecular weight determination with calibrated columns; (c) desalting, buffer exchange and removal of low molecular weight products such

Table 1-2: Gel Filtration Media and Some of Their Properties for Separation of High Molecular Weight Molecules.

Medium	Matrix	Fractionation range		Supplier
		proteins	polysaccharides	
Bio-Gel	agarose			Bio-Rad Lab.
A-1.5m		1×10^4... 1.5×10^6	–[a]	
A-5m		1×10^4... 5×10^6	–	
A-15m		4×10^4... 15×10^6	–	
A-50m		1×10^5... 50×10^6	–	
A-150m		1×10^6...150×10^6	–	
Sepharose	agarose			Pharmacia Fine
2B/CL-2B		7×10^4... 40×10^6	10^5...20×10^6	Chemicals
4B/CL-4B		6×10^4... 20×10^6	3×10^4... 5×10^6	
6B/CL-B		1×10^4... 4×10^6	10^4... 1×10^6	
Ultrogel	agarose			LKB Produkter
A2		1×10^5... 2×10^7	–	
A4		5×10^4... 9×10^6	–	
A6		2×10^4... 2×10^6	–	
Bio-Gel	acrylamide			Bio-Rad Lab.
P-300		6×10^4... 4×10^5	–	
Ultrogel	acryl./agarose			LKB Produkter
AcA 22		1×10^5... 1×10^6	–	
AcA 34		2×10^4... 3×10^5	–	
Sephadex	dextran			Pharmacia Fine
G-200		5×10^3... 6×10^5	10^3... 2×10^5	Chemicals
Sephacryl	dextran/acryl.			Pharmacia Fine
S-200		5×10^3... 2.5×10^5	1×10^3... 8×10^4	Chemicals
S-300		1×10^4... 1.5×10^6	1×10^3... 4×10^5	
S-400		2×10^4... 8×10^6	1×10^4... 2×10^6	
S-500		–	4×10^4...20×10^6	
S-1000		–	5×10^5... $>10^8$	

[a] No data available

as unconjugated labels e.g. FITC, inhibitors etc.; (d) fractionation of molecular subunits after detergent or enzyme treatments. Gel beds are suspended in a selected buffer and poured into a column. Gel particles represent the so-called stationary phase and their solvent (buffer) is in equilibrium with the solvent outside the gel. Large molecules (larger than the largest pores) do not penetrate stationary phase and migrate only in the mobile phase as the fastest fraction through the column (Fig. 1-14); they are eluted with the column void volume (V_o). According to their size the smaller molecules diffuse into the stationary phase and are eluted from the column in order of decreasing molecular size. The results of a chromatographic run are expressed in the form of an elution diagram detected by continuous UV-monitoring and registration which allows the determination of the elution volume (V_e). For theoretical considerations of solute behavior in gel filtration see Porath [51], Laurent and Killander [52] and Determann [53]. Selection of gels according to K_{av} values of macromolecules, specifications and technical details for the preparation of gel filtration columns are given in the manufacturers' instruction manuals.

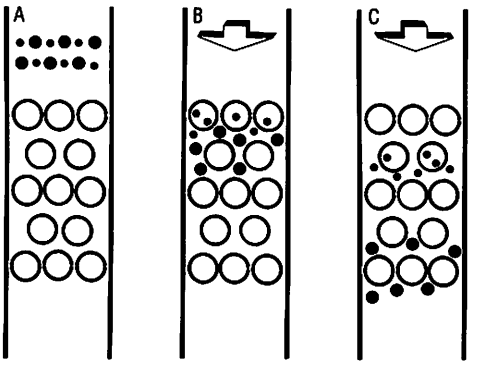


Figure 1-14. Principle of gel filtration; application of small and large molecules onto a gel bed (A); small molecules diffuse into the stationary phase, large molecules migrate only in the column void volume (B, C).

For our second purification step, samples obtained from DEAE ion exchange chromatography at 0.01 mol/L to 0.3 mol/L NaCl gradients, respectively, are submitted to gel filtration. The experiment is as follows.

Protocol of gel filtration:

1. Preparation of the column: Sephadex G-200 is swollen and equilibrated with PBS at 4 °C, then poured into a column (100 x 2 cm bed size) and prepared for downward elution.
2. Calibration: the column throughflow is registered continuously at $A_{280\,nm}$ and calibrated with substances of known molecular weight. Individual fractions of 10 mL each are collected.
3. Gel filtration: the respective DEAE peaks are finally applied; Fig. 1-15 shows a typical elution profile obtained with DEAE peak at 0.1 mol/L NaCl and measured at $A_{280\,nm}$. Individual fractions are further examined for PAS reacting substances by a colorimetric method [46] and data are incorporated into the curve. Individual fractions of the obtained gel filtration peaks are then pooled, concentrated to 5 mg/mL protein and dialyzed against PBS.

It can be seen in Fig. 1-15 that the bulk of PAS staining molecules elute with the void volume of the column (first peak). These molecules possess molecular weights of > 150000 daltons according to the calibration characteristics of the column. The second large gel filtration peak contains only small amounts of PAS positive substances. In analytical PAA electrophoresis, each of the gel filtration peaks give several PAS positive zones. Moreover, fur-

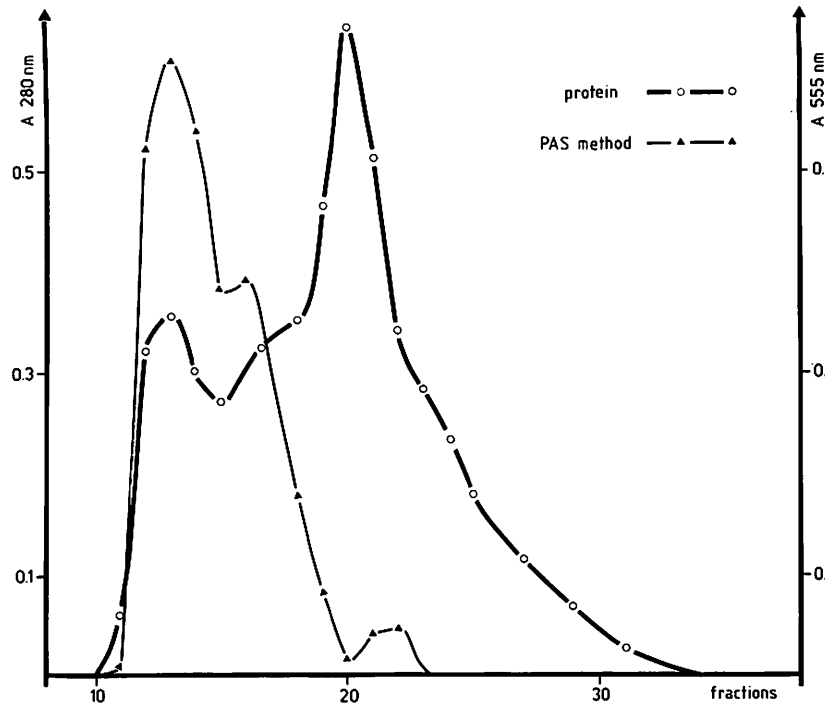


Figure 1-15. DEAE peak obtained with 0.1 mol/L NaCl is submitted to Sephadex G-200 gel filtration; elution profile, see text.

ther zones which stain for proteins are found, too. In comparison to DEAE ion exchange chromatography, no significant progress was achieved in the purification of neutral glycoconjugates from extracts of human stomach by use of Sephadex G-200 gel filtration. The latter step may be omitted. In the course of isolation of high molecular weight PAS positive glycoconjugates, DEAE gradient fractions or the V_0 fractions of the Sephadex G-200 gel filtration experiment must be submitted to further purification steps. For our material, the next choice is chromatofocusing.

1.2.3 Chromatofocusing

Preparative purification of gastric mucosa glycoproteins can also be tried by chromatofocusing. This method separates molecules according to their isoelectric points and focusing effects are produced by an internal pH gradient in an ion exchange chromatography column. The charged groups of an ion exchanger have a buffering capacity, and the internal pH gradient is obtained by passing a buffer of a certain initial pH (e.g. pH 4.0) through the ion exchange column pre-adjusted to a different pH (e.g. pH 7.4). The formed pH gradient is able to elute protein molecules being bound to the ion exchanger, and elution is in order of their isoelectric points. Principles of isoelectric focusing in ion exchange columns are described by Sluyterman and Elgersma [54, 55].

Chromatofocusing is performed in columns filled with ion exchangers adjusted to a higher pH than the eluant buffer used (descending gradient). The protein sample is put on top of the column and the protein net charge determines its binding to the ion exchanger, e.g. an anion exchanger (Fig. 1-16). The net charge of a protein is known to depend on the environmental pH and is zero at a specific pH which is called the isoelectric point pI (see 1.2.1). In the course of chromatography, a pH gradient from the column pH to eluant buffer pH is formed. When the pH drops below the isoelectric point, the protein becomes positively charged and migrates down the positively charged anion exchanger with e.g. the applied elution buffer of pH 4.0. In the course of protein migration down the column along with the eluant buffer, the latter becomes buffered at a given moment to a higher pH by the ion exchanger, and, consequently, the pH rises above the pI of the protein and it reverses its charge and binds to the ion exchanger. In the further steps of elution, the developing pH gradient again causes the pH to drop below the pI of the protein which then again migrates down the column. This process is repeated until the pH gradient has progressed through the column and the protein is finally eluted. When the sample applied to the chromatofocusing column contains proteins with different pIs, these will migrate different distances on the ion exchangers before their binding. The elution is then in order of their isoelectric points.

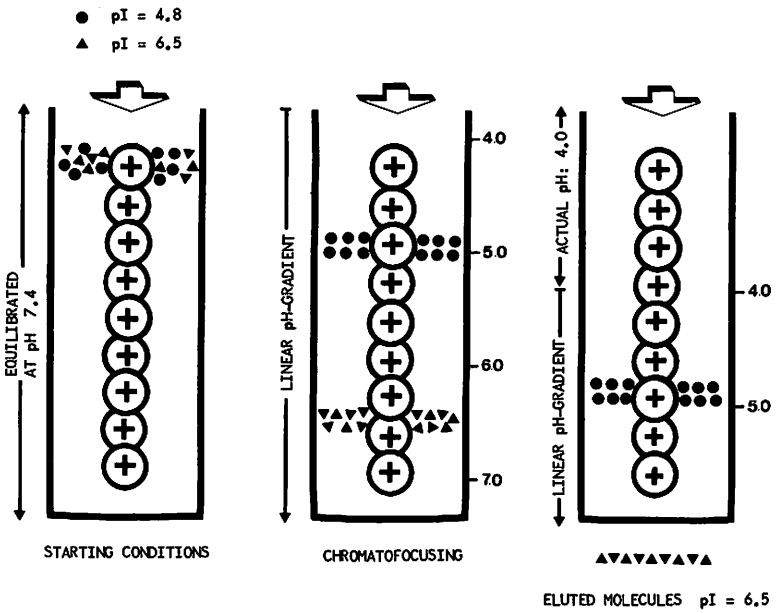


Figure 1-16. Principle of chromatofocusing, see text.

In order to purify gastric mucosa glycoproteins, samples from DEAE ion exchange chromatography and gel filtration are further processed for chromatofocusing in bead formed gels by use of commercial ion exchangers (Polybuffer exchanger PBE 94) and amphoteric buffer (Polybuffer 74) both obtainable from Pharmacia Fine Chemicals (Uppsala, Sweden); specifications and technical details of chromatofocusing are given in the respective instruction manuals.

Protocol of chromatofocusing:

1. Preparation of the column: PBE 94 which is based on Sepharose 6B and covers the range pH 9 to 4 is equilibrated with 0.025 mol/L imidazole-HCl buffer pH 7.4 and poured into an appropriate column.
2. Sample preparation: The sample is equilibrated with eluent buffer by dialysis. The Polybuffer 74 (Polybuffer 74-HCl pH 4.0) is chosen for the range pH 7 to 4. Generally, Polybuffers are manufactured in such a way as to give an even buffering capacity over the indicated pH range. Thus, the upper limit of the gradient in the chromatofocusing column is determined by the column pH (i.e. pH 7.4) and the lower limit of the gradient is defined by the pH of the eluent buffer (i.e. pH 4).
3. Chromatofocusing: First, 5 mL of eluent buffer are run in the column, then the sample is applied and followed by eluent buffer. The column throughflow is continuously monitored at $A_{280\,nm}$ (Fig. 1-17). Individual fractions of eluted peaks are pooled and Polybuffer is removed by gel filtration on Sephadex G-75. Finally, peaks are concentrated to 5 mg/mL protein by ultrafiltration and dialyzed against PBS.

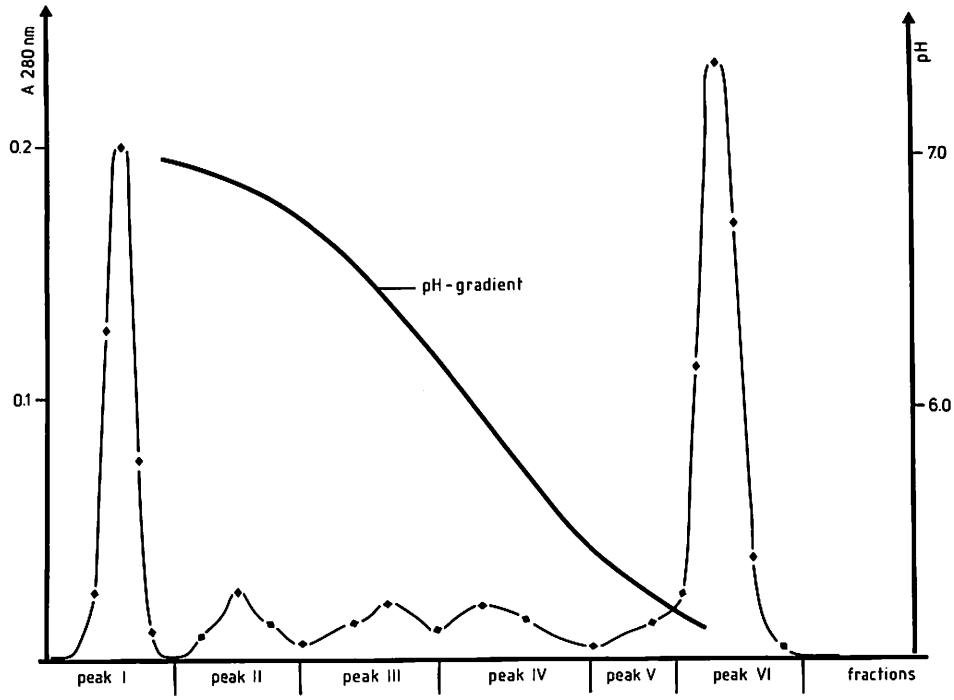


Figure 1-17. Elution profile of chromatofocusing of the DEAE peak obtained at 0.01 mol/L. It is seen that this peak is further fractionated (Peaks I to VI) which corresponds to different pI of the molecules.

The purification efficiency of the different peaks (molecules with different pI) is then examined by zone electrophoresis. As described above for DEAE ion exchange chromatography, one plate is stained for proteins and another plate is stained with PAS substrate (not shown). From our experience we do not expect homogeneity within a given peak. Indeed, analytical PAA gel electrophoresis reveals several PAS reacting zones for each of the different collected peaks. Thus, the next step assumed to result in high quality purification is to submit individual peaks to preparative electrophoresis.

1.2.4 Preparative polyacrylamide gel electrophoresis

Ever since electrophoretic methods have been available, such techniques are used for the preparative isolation of molecules. The same principles which are described above for the evaluation of antigens and antibodies by analytical electrophoresis hold true for preparative purposes, and PAA gels due to their reproducibly high resolution are of special interest. The basis for optimal preparative electrophoresis of molecules is the technical adaption from previous analytical electrophoresis; theoretical considerations have already been given (see Chapter 1.1.1 and references [56 – 58].

Preparative electrophoresis in PAA gels proved to be a useful tool for the isolation of molecules like AFP [59] and intestinal goblet cell antigen (GOA) [60]. In the course of purification of gastric mucosa neutral glycoproteins, this approach can also be successfully applied. Preparative electrophoresis is performed in a horizontal PAA gel slab by use of a rectangular quartz glass chamber with interior dimensions of 18 cm x 9 cm x 0.3 cm. Connections with the electrode compartments are assured by gel bridges which are cast with the separation gel (for anodic junction) and after sample application (for cathodic junction), respectively. For electrophoresis of samples, a separation gel of 8 % PAA with a large-pore spacer gel of 3.5 % PAA for sample concentration is used. Reproducible electrophoresis can be only achieved with effective cooling of the gel. The latter is reached by contact of the whole separation chamber with a polished metal plate being cooled with Peltier elements to 4 °C. After electrophoresis, gel strips of 3 mm each are cut with a nylon thread on a standard cutting device. Then, individual strips are submitted to a freeze-thaw cycle and homogenized, followed by combined elution and concentration by use of vacuum ultrafiltration in dialysis bags [61].

A view of the electrophoretic unit with the elution/concentration apparatus and a schematic drawing of the preparative PAA separation chamber is shown in Fig. 1-18.

In typical preparative experiments, peaks from the above chromatofocusing are submitted to preparative electrophoresis. In the present example, Peak I (eluted at pH 7.27) is chosen for demonstration.

Protocol of preparative PAA electrophoresis:

1. Preparation of the gel slab: separation gel and anodic junction gel consist of 8 % PAA in 0.175 mol/L Tris-citric acid pH 9.0 which is cast into the vertically positioned glass chamber and covered with a spacer gel of 3.5 % PAA in 0.023 mol/L Tris-citric acid pH 9.0. Then, the sample slot former (8 cm x 1.5 cm) is inserted.
2. Sample application: after polymerization, the sample slot former is removed. The pocket is first filled with cathodic junction gel (3.5 % PAA in 0.065 mol/L Tris-boric acid pH 9.0), then the sample (1 mL containing about 10 mg protein) is placed underneath with the help of a syringe. In the present example, the sample is a mixture of chromatofocusing peak I (9 volumes) and ethylene glycol (1 volume) with a small amount of bromphenol blue as tracking dye.
3. Electrophoresis: finally, the cathodic junction gel (3.5 % PAA in 0.065 mol/L Tris-boric acid pH 9.0) is cast and polymerized in horizontal position; electrode buffers are 0.065 mol/L Tris-boric acid pH 9.0. Electrophoresis is run with 350 volts under constant cooling at 4 °C. When the bromphenol blue tracking has migrated 17 cm towards the anode, gel strips of 3 mm are cut, eluted and concentrated as described above.

The purification efficiency of preparative electrophoresis is finally examined by analytical zone electrophoresis in PAA. When PAS staining neutral glycoproteins obtained in

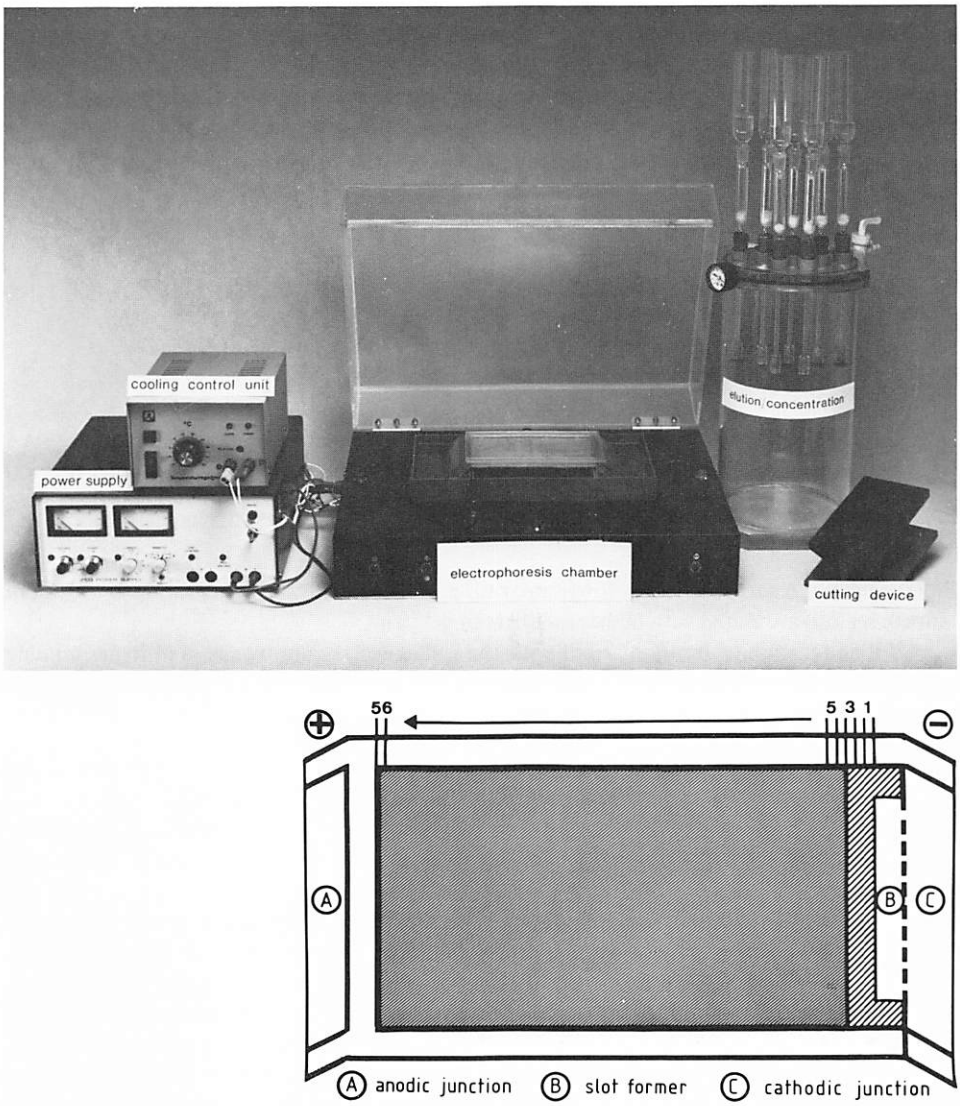


Figure 1-18. Working place for preparative PAA electrophoresis with apparatus for elution and concentration of gel strips (a); schematic drawing of the electrophoresis chamber (b).

peak I of the chromatofocusing experiment are submitted to preparative PAA electrophoresis, we observe in analytical PAA electrophoresis (Coomassie Blue and PAS stainings of respective gel plates) that preparative electrophoresis is able to fractionate the apparent homogenous peak I (homogenous with respect to pI) into single zones. Neutral glycoproteins are eluted from cathodic and anodic gel strips. In Fig. 1-19, a selection of slowly migrating (more cathodic region) molecules is shown. At least some of them, i.e. the designated numbers 5, 7, 8, 9 and 10 which correspond to the numbers of eluted gel strips, possess apparent

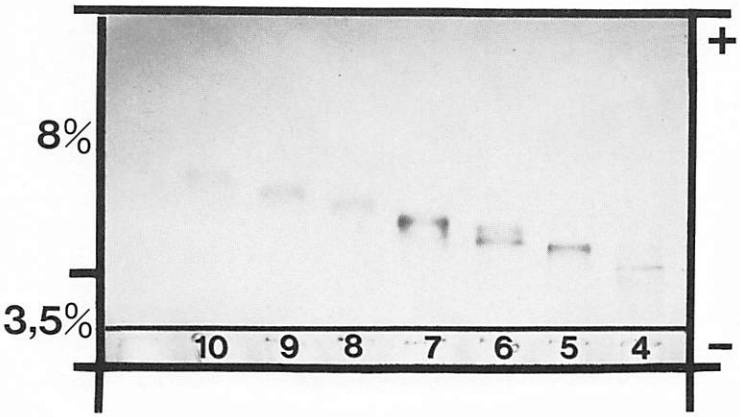


Figure 1-19. Analytical PAA electrophoresis of PAS staining molecules obtained by preparative PAA electrophoresis. Starting materials are PAS molecules present in peak I from chromatofocusing (pI 7.27) described above. Fraction numbers 4 to 10 correspond to eluted cathodic gel strips. Various PAS positive zones of different mobility are seen; PAS reaction.

homogeneity. Apart from the slowly migrating glycoproteins, fast migrating ones (more anodic region) are eluted with higher numbers of gel strips.

Finally, rabbits are immunized with the isolated glycoconjugates, and hyperimmune sera are employed for subsequent immunological and immunohistological characterization of gastric mucosa in normal and diseased states (see also Chapter 5.2).

1.2.5 Affinity chromatography

Bioselective affinity chromatography is presumed to be the most powerful separation technique because it is based on functional (which includes particular chemical structure) rather than physicochemical properties (like positive or negative net charges) of the biomolecules. To this end, a biospecific ligand is first attached to an inert and insoluble matrix, and the molecules to be purified are selectively and reversibly adsorbed to the immobilized ligand. The first use of affinity chromatography dates back to 1910 when Starkenstein [62] employed insoluble starch for selective adsorption of amylase. The introduction of cyanogen bromide activated polysaccharide matrix for the coupling of molecules through their primary amino groups [63] was a breakthrough for widespread application and in the meantime, affinity chromatography has become a routine technique in biochemical and immunochemical laboratories (review at Scouten [64]). Thus, adsorbents for

- groups of related substances (e.g. protein A adsorbent with affinity for Fc region of IgG molecules),
- single substance (e.g. immunoadsorbent with affinity for specific antigen or antibody),
- hydrophobic interactions (e.g. immobilized hydrophobic groups for hydrophobic interaction chromatography)

can be prepared. A variety of coupling gels for ligand immobilization and ready to use group specific adsorbents for affinity chromatography are now commercially available.

For selective separation of molecules by affinity chromatography, a highly specific ligand with reversible binding affinity for the molecules to be isolated must be available which possesses chemically modifiable groups to be covalently coupled to an insoluble support. For the latter, bead-formed agarose (Sepharose, Pharmacia, Uppsala, Sweden) proves very valuable because its hydroxyl groups can be readily derivatized for covalent coupling of a ligand. For the isolation of immunologically reactive molecules, we can profit from the high selectivity (specificity) and the high affinity of the antigen-antibody reaction. Thus, antigens or specific antibodies may be coupled to agarose beads for the selective isolation of antibodies or antigens, respectively. For this approach, cyanogen bromide (CNBr) activated agarose beads are prepared [65] which enable all kinds of proteins and ligands containing primary amino groups to be immobilized quickly and easily (Fig. 1-20). CNBr reacts with hydroxyl groups of agarose by potential formation of imidocarbonates and cyanate esters. These activated groups will then form isourea linkage with amino groups of ligands (e.g. specific antibodies) added in a second step. In this example, the immobilized ligand is used as an immunoadsorbent by reversible binding of its antigens which are added in a third step. Molecules with no or other affinities are washed off from the immunoadsorbent, and desorption of specific molecules is finally obtained by treatment of the immunoadsorbent with chaotropic ions.

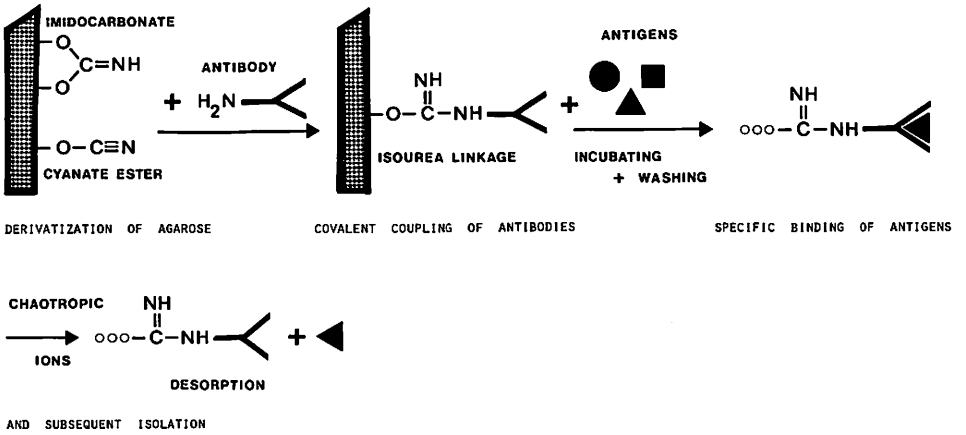


Figure 1-20. Immunoadsorption with covalently coupled antibodies to CNBr activated agarose beads, see text.

Solid-phase immunoadsorption will often allow separation of biomolecules in a single assay. Yet, such a selective purification procedure was not possible at the beginning of our experiments because a specific immune serum against our desired gastric mucosa glycoproteins was not available. In order to demonstrate feasibility of the method we describe now the isolation of rat AFP from amniotic fluid; a specific immune serum against rat AFP was available.

Protocol of preparation and use of an immunoadsorbent:

1. Gel activation: Sepharose 4B beads (1 mL settled gel/5 mg protein) are washed with distilled water and activated with CNBr (0.2 g/1 mL gel) at pH 11 (temperature: below 20 °C).
2. Coupling of ligand: in the case of antibody coupling, activated gel is washed with ice-cold 0.15 mol/L phosphate buffer pH 6.8 containing 0.5 mol/L NaCl. Then, immunoglobulin fractions of rabbit anti-rat AFP immune serum are added (5 mg/1 mL gel) and put on a rotary shaker for 18 hours. The product is washed on a Büchner funnel with excess of ice-cold PBS and remaining active groups on the gel are blocked by overnight suspension in 0.2 – 0.4 mol/L ethanolamine-HCl buffer pH 7.4. In order to remove uncoupled and ionically bound ligands, the immunoadsorbent is treated alternately with excess of low and high pH buffers, (a) 0.1 mol/L acetate buffer pH 4.0 plus 0.5 mol/L NaCl, (b) 0.1 mol/L carbonate buffer pH 8.0 plus 0.5 mol/L NaCl and followed by 3 mol/L NaSCN in PBS (desorption buffer) and PBS alone; all procedures at 0 – 4 °C. About 90 % of the employed ligands are coupled to the agarose matrix.
3. Affinity chromatography: the immunoadsorbent is packed into a column and equilibrated with PBS or with 0.1 mol/L phosphate buffer pH 7.2 plus 0.4 mol/L NaCl and 0.2 % Tween 80. Eveleigh and Levy [66] found that Tween included in all buffers eliminates non-specifically bound proteins. Then, the column is run with the probe, i.e. AFP rich amniotic fluid of rat, and subsequently washed with excess of starting buffer; the addition of NaCl in molarities up to 1 or 2 mol/L buffer proved to be useful for removing non-specifically bound proteins [59]. Finally, ligand-bound molecules are eluted by use of chaotropic ions at concentrations of 3 mol/L buffer [59, 67]; we desorb by reverse flow direction of the column. The eluate is immediately passed on a Sephadex G-25 fine column equilibrated with PBS in order to remove NaSCN from the antigens (Fig. 1-21). Isolated AFP is concentrated to 3 – 5 mg/mL protein by vaccum ultrafiltration and dialyzed against PBS.

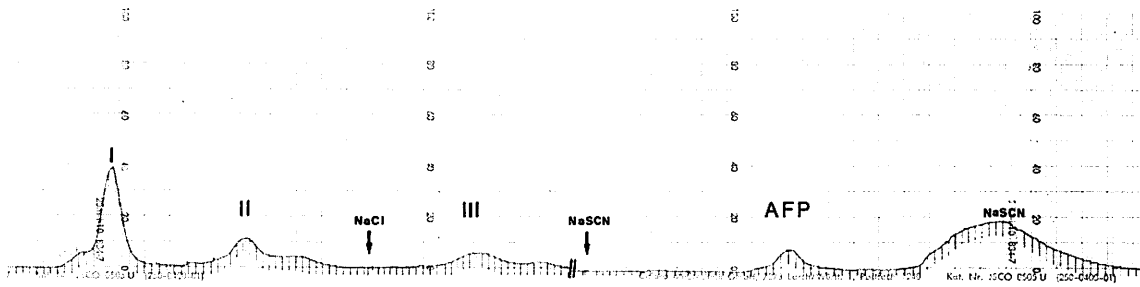


Figure 1-21. Immunoadsorption of AFP from amniotic fluid by use of immobilized anti-AFP. Column throughflow is registered at $E_{280\ nm}$; non-bound proteins (I and II) and non-specifically bound molecules (III) are washed from the column with starting buffer and 2 mol/L NaCl. Ligand-bound AFP (AFP) is desorbed with 3 mol/L NaSCN which appears as last peak when passed on Sephadex G-25 gel.

The purity of isolated AFP molecules is examined by analytical zone electrophoresis in PAA gels and by immunoelectrophoretic as well as double diffusion in gel tests. Results are shown in Chapters 1.1.1 and 1.1.2.

The controls described above (Chapter 1.) on the purity of the antigen/antibody preparations are satisfactory for immunohistological work inasmuch as in a first experimental approach the isolated molecules are taken for immunization of animals. It is readily understood that isolated substances may be further characterized by a variety of biochemical and immunological methods. The latter are necessary in order to obtain detailed information about structural and functional heterogeneity, the description of which is not within the scope of the present work.

1.3 Preparation of Immune Sera and Antibodies

In the past, the cellular basis for antibody formation was the subject of several theories, e.g., the selective theory according to Ehrlich [68], the instructive antigen-template theories as suggested by Pauling [69] and Breinl and Haurowitz [70] and the clonal selection theory by Burnet [71]. There is now sufficient evidence from numerous experiments to support the clonal theory. Immune sera for experimental work are usually obtained from mammals or fowl, and rabbit immune sera are the most frequently employed. Whether immune sera contain precipitating or non-precipitating antibodies does not affect their usefulness for immunocytochemical work.

1.3.1 Lymphoid cells and antibody production

The immune system is a diffuse organ dispersed in the body and basically constitutes lymphoid cells and antibody molecules. The latter are the product of the lymphoid cells. The task of the whole immune system is maintenance of the integrity of the organism. Defects or irregularity of the immune system involves a large number of diseases ranging from autoimmune disease to cancer, allergy and other disorders including aging [72]. The ability of a substance to cause specific antibody production when introduced into a host is called immunogenicity, and we profit from such an immunogenic effect for the experimental preparation of immune sera by immunization of animals with isolated antigens.

Lymphocytes in the various lymphoid tissues have come from a stem cell pool in the bone marrow and have passed either through the thymus (T cells) or an organ system equivalent to the Bursa of Fabricius (B cells). B cells and their progenitors are responsible for the synthesis and secretion of humoral antibodies (review by Nossal and Ada [73]). Biosynthesis of antibodies is regulated by the immune system by cooperation of the various lymphoid cell-types and involves recognition and processing of the antigenic/immunogenic information and blast cell transformation of committed lymphocytes. Finally, antibody genes are transcribed into messenger RNA which interact with ribosomes, enabling the competent cells to synthesize antibody molecules.

Reconstructions of ultrastructural events from the time of immunization to the recognition of specific antibodies have been attempted with ferritin labelings [74], enzyme labelings [75, 76], plaque- and rosette-forming tests [77 – 79]. Immunoenzyme techniques proved especially useful for the cellular localization of specific antibodies at light and electron microscopic levels (see references [80, 81]). During both primary and secondary immune responses, intracellular localization of antibody is not observed in typical small lymphocytes. Labelings are attained parallel with the development of the rough surfaced endoplasmic reticulum (RER), the characteristic sign of differentiating blast cells. Then, antibodies occur in the perinuclear space, in lamellae of the RER and in the Golgi apparatus of immunocytes (Fig. 1-22). In the course of maturation, antibody synthesis proceeds to its full extent. In parallel, the Golgi complex becomes very prominent of which the lamellar and vesicular systems are heavily filled with antibody suggesting secretion via the Golgi apparatus.

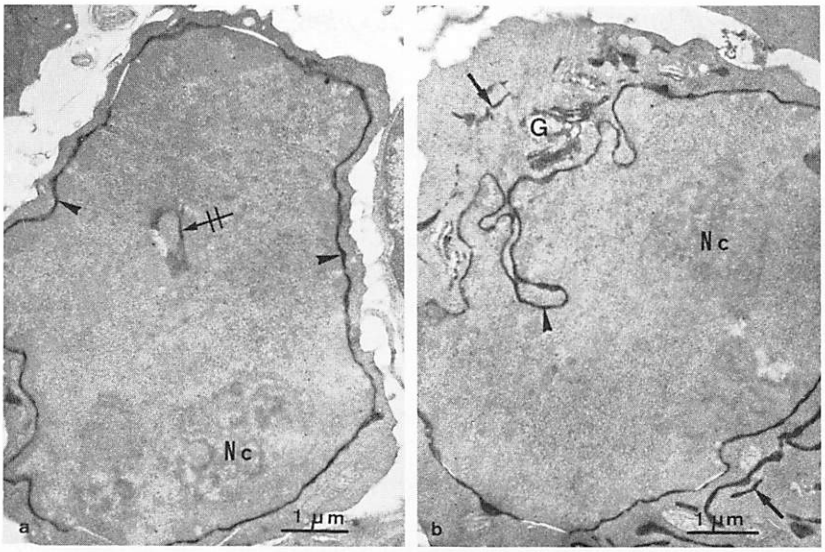


Figure 1-22. Synthesis of specific antibody in RER lamellae (←) and the PNS (◂) of blast cells (a and b). The Golgi apparatus (G) is also stained. Note deep invaginations of the PNS which is often cut tangentially (◂‖); nucleolus (Nc). Lead salt counterstain (from W.D. Kuhlmann, *Prog. Histochem. Cytochem.* **10/1** [1977] 1).

1.3.2 Hyperimmunization of animals: polyclonal and monoclonal antibodies

The development of a reliable immuno-assay depends on immune sera with appropriate titer, specificity and affinity. The titer may be defined as the final dilution used in the assay. Specificity is the degree of uniqueness with which the antibodies bind the substance assayed. Affinity may be defined as the avidity with which the antibody binds the antigen. No absolute criteria exist; for experimental details see references [6-8, 82, 83].

When preparing antibodies by immunization of animals, one can obtain a broad spectrum of specific antibodies against the immunizing antigen: i.e. antibodies against the different epitopes which occur in the antigen. These antibodies are derived from different clones, and each of them has originated from a committed lymphocyte. The immunization of an animal with a given antigen will, thus, give rise to the production of multiple antibody populations which recognize the different antigenic components of the used immunogen. Despite specificity for a single antigenic determinant, the antibodies in the animal serum are heterogenous inasmuch as an identical combination of specific antibodies cannot be obtained upon immunization of a second animal with the same immunogen.

The recently introduced hybridoma technique, i.e. the fusion of immunocytes each committed to the synthesis of a single antibody species with myeloma cells, and their subsequent cloning [84] is a powerful approach for the reproducible production of monoclonal antibodies. Such hybrid myeloma cell lines can be maintained unlimited (e.g. *in vitro*) and produce antibodies of uniform specificity. The production of monoclonal antibodies in unlimited quantity is a biotechnology of great importance. However, the monoclonal antibody technique has not yet reached the point to be applied routinely for the preparation of all

immune sera. Moreover and very importantly, most tissue specimens for immunohistological studies must be stabilized by chemical fixatives which can denature at random and in uncontrollable manner a great deal of the present cellular antigens (cf. Chapter 4.1). Because a monoclonal antibody characterizes by definition only one antigenic determinant from the antigen spectrum primarily applied to the animal by immunization, it is possible that in the final immunohistological assay the defined monoclonal antibody will not recognize its antigen. From this can be deduced that it is not always preferable to employ monoclonal antibodies for immuno-staining of fixed antigen in tissue preparations. Hence, immunization of animals for obtaining polyclonal antibodies in immune sera is not obsolete.

In humans, immunoglobulins may occur in five major classes: IgG, IgM, IgA, IgD and IgE. The immunoglobulins are found (human or animals) in the γ-globulin fraction which is called as such according to its electrophoretic mobility. In hyperimmune sera which we used throughout the work described here, specific antibodies are almost all IgG globulins. The structure and shape of IgG molecules have been studied extensively [85 – 89]. Briefly, the IgG globulin has a molecular weight of about 150000 daltons and consists of 4 polypeptide chains; 2 identical heavy or H chains and 2 identical light or L chains (Fig. 1-23). All chains possess constant and variable regions, but only the variable regions account for the specificity of the antibody reaction, i.e. the sites which bind specifically with the antigen. When an IgG globulin is treated with papain, it splits into three parts: one Fc fragment which is inactive in antigen binding and two Fab fragments, each containing one combining site (MW 40000). Upon pepsin treatment, the two Fab units are left united in a bivalent fragment while cleaving

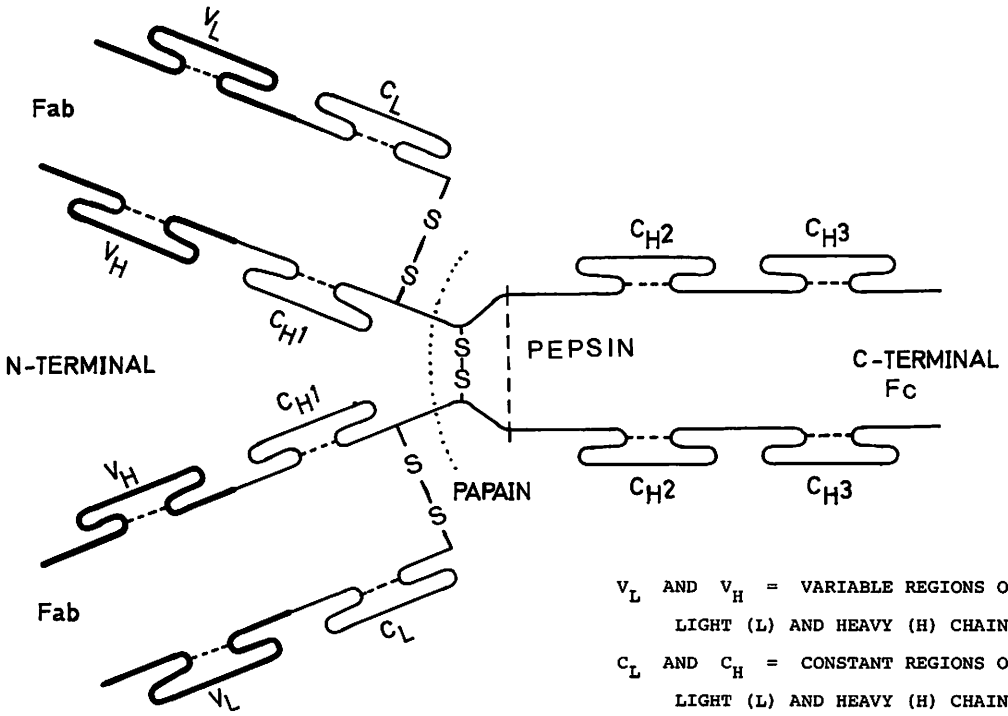


Figure 1-23. Structure of IgG immunoglobulin, see references [86, 89, 90].

the Fc fragment. After reduction with mercaptoethanol, the Fab'_2 splits into two univalent fragments.

High titer antibodies are readily obtained when primed animals are boostered with the same antigen. A typical experiment is shown in Fig. 1-24 and in Fig. 1-25 where circulating antibodies and numbers of antibody synthesizing immunocytes are determined by serological and immunocytological techniques after a single stimulation and after a booster injection with a given immunogen (horseradish peroxidase in this example) into the popliteal lymph nodes.

Numerous pitfalls can be encountered in the preparation of immune sera. During immunization with proteins, relatively minor contaminants (and depending on their immunogenicity) in the immunizing mixture may produce more antibody than the primary immunogen. The careful analysis of immune sera by methods like immunoelectrophoresis, the Ouchterlony test and immunohistology and after various schedules of immunological absorption with different antigen preparations and possible contaminating macromolecules will permit the identification of unwanted antibodies.

Natural antibodies in sera may lead to immunohistological staining reactions which are difficult to judge. Thus, previous antigenic stimulation e.g. with infectious organisms which share common antigenic determinants with the studied material will give rise to such natural antibodies. Natural antibodies are also those with anti-A and anti-B reactivities in normal human sera. Furthermore, cross-reacting antibodies can arise upon immunization. Cross-reactions may be due to certain antibody heterogeneity as a result of immunization of an animal with an antigen containing different types of antigenic determinants. This is the case when for example molecules used for immunization possess in addition to their "specific" antigenic determinants further determinants which are also shared by other molecules in other

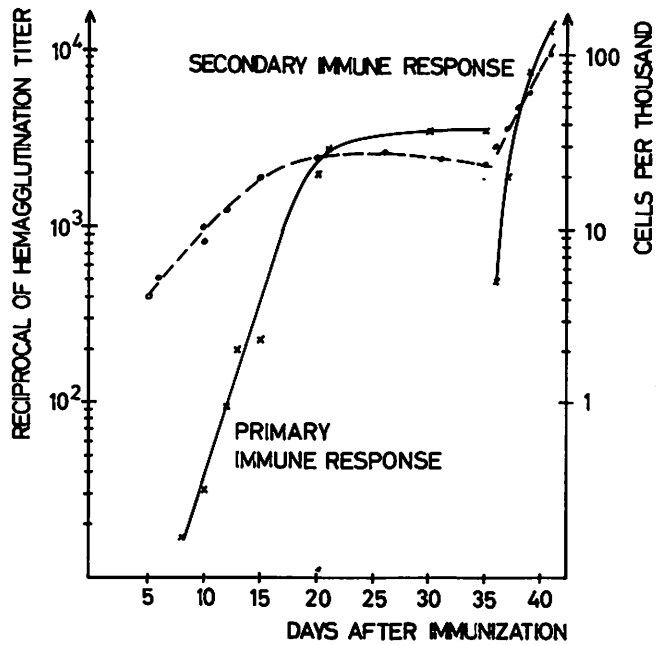


Figure 1-24. Hemagglutination titer of circulating anti-peroxidase antibodies (x———x) and number of anti-peroxidase positive immunocytes (o-----o) in lymph node cell suspensions during primary and secondary immune response (from W. D. Kuhlmann, *Prog. Histochem. Cytochem.* **10/1** [1977] 1).

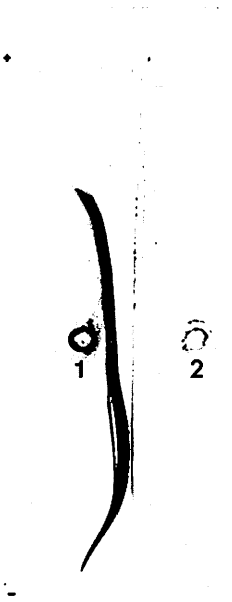


Figure 1-25. Immunoelectrophoretic analysis of a rat hyperimmune serum against peroxidase (1) and a normal non-immune rat serum (2); after electrophoresis in agar, the plate is reacted with peroxidase, washed in PBS, air-dried and stained for peroxidase activity. Note that antibodies against peroxidase are present in the hyperimmune serum which are almost immunoglobulins of the IgG class; normal non-immune serum (2) contains no antibodies against peroxidase.

tissues. Cross-reactivity of antibodies can also be due to similar antigenic determinants shared by different antigen molecules in different organs. In the following case, a rabbit is immunized with a tissue antigen I which carries e.g. two antigenic determinants (Fig. 1-26). Then, two antibody species can be elicited and upon incubation with homologous tissue I,

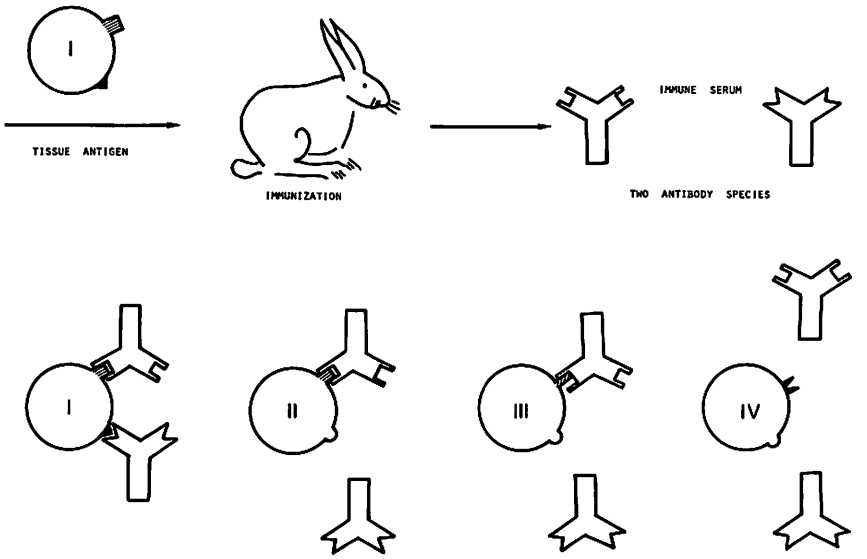


Figure 1-26. Incubation of four different tissues (I to IV) with two antibody species.

both antibodies will react. In a heterologous tissue II, an antigen may carry one of the two antigenic determinants, thus, one species of the antibodies will react. In another case III, a tissue antigen may possess a similar but not fully identical determinant so that it can be recognized by the virtually different antibody, and, thus, giving also an immunological reaction. Finally, a tissue IV may possess antigens in which neither of the above antigenic determinants is present, and, consequently, both antibody species will not react. For theoretical aspects and experimental details on the cross-reactivity of antibodies see Eisen [91].

In the course of immunization, considerable variations in specific antibody levels can be observed in individual animals. Furthermore, there are great differences from one laboratory to another in the method of production of their immune sera. Here, we present our own immunization schedules which are modifications from previously described methods [92] and with which we obtain reliable results.

Protocol of immunization of rabbits:

1. Primary injection: 0.5 mg of pure antigen per rabbit (young adult) is used. The antigen is dissolved in 0.5 mL saline and emulsified with 0.5 mL complete Freund's adjuvant. Rabbits are immunized into both hind foot pads by intracutaneous injections.
2. Booster injections: the first booster injection is done one month after primary immunization. The same amount of antigen is dissolved in 0.5 mL saline and emulsified with 0.5 mL incomplete Freund's adjuvant. Rabbits are immunized intramuscularily into the left and right leg muscle and subcutaneously at two different sites in the back. Further booster injections may follow and are usually done at monthly intervals. Animals are bled one week after the last injection.

Protocol of immunization of sheep:

1. Primary injection: 1 mg of pure antigen per sheep (young adult) is used. The antigen is dissolved in 5 mL saline and emulsified with 5 mL complete Freund's adjuvant. Animals are immunized subcutaneously at 10 different sites in the back.
2. Booster injections: booster injections are done after 4 weeks with 0.5 mg antigen dissolved in 4 mL saline and emulsified with 4 mL incomplete Freund's adjuvant. Sheep are immunized subcutaneously at 4 different sites in the back (shoulder and hip region). Booster injections are repeated at monthly intervals for a period of 6 – 8 months. Following this immunization schedule, the amount of antibody in sera reaches its highest level (Fig. 1-27). Then, sheep may be bled or further boostered.

Prior to employing the immune sera for specific use, these are qualitatively and quantitatively examined by gel diffusion techniques, e.g. the Ouchterlony test, immunoelectrophoretic analyses and single radial immunodiffusion; technical details are given in Chapter 1.1. We must be aware that undetectable traces of plasma proteins or unrelated cellular constituents may contaminate our isolated antigens which may also elicit antibodies in animals. Hence, those antibodies must be eliminated by subsequent absorption. This is done by solid-phase immunoadsorbents prepared from normal plasma proteins and tissue extracts. Even if the produced immune sera do not show immunological reactivity by *in vitro* testing with normal plasma proteins or with extracts from unrelated cells, such immunoabsorption is regularly performed in order to render the immune serum as specific as possible.

1.3.3 Isolation of antibodies by affinity chromatography

The use of isolated antibodies out of the whole immune serum is of importance for immunohistological work of highest specificity. Light and especially electron microscopical

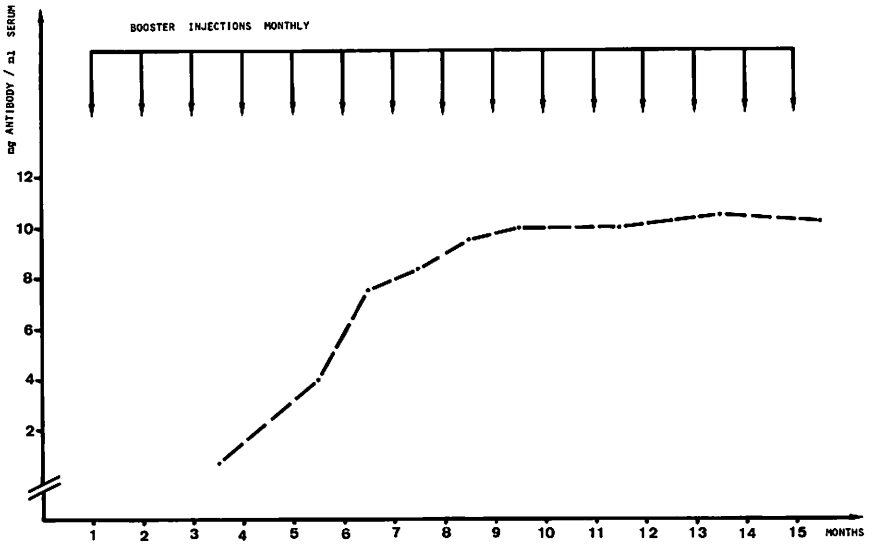


Figure 1-27. Amounts of antibodies (e.g. anti-rabbit IgG) in sheep sera during immunization.

immuno-stainings have shown that whole immune sera or even γ-globulin fractions (e.g. obtained by DEAE ion exchange chromatography) can provoke non-specific stainings [43, 59, 93].

Purification of specific antibody molecules from complete immune sera is possible by use of water-insoluble immunoadsorbents which can be obtained by covalent coupling of antigens to solid supports [94, 95]. Two techniques in particular have prevailed: (a) immunoadsorption with insolubilized antigens using glutaraldehyde as cross-linking reagent [96] and (b) immunoadsorption with covalently coupled antigens to cyanogen bromide activated agarose beads [63, 65]. Personally, we prefer the latter approach; its principle is described in Chapter 1.2.5.

Protocol of preparation and use of immunoadsorbent:

1. Gel activation: Sepharose 4B is activated with cyanogen bromide as described above.
2. Coupling of ligand: in the case of antigen coupling, activated gel is washed with ice-cold 0.1 mol/L carbonate buffer pH 8.3 containing 0.5 mol/L NaCl. Then, antigen (e.g. rabbit IgG) is added to the gel (5 mg protein per mL gel) and put on a rotary shaker for 18 hours.; subsequent washings are as described in Chapter 1.2.5.
3. Affinity chromatography: the immunoadsorbent is packed into a column and equilibrated with 0.1 mol/L phosphate buffer pH 7.2 plus 0.4 mol/L NaCl and 0.2 % Tween 80. Then, the column is run with sheep anti-rabbit IgG immune serum and subsequently washed with excess of starting buffer; further procedures are as described in Chapter 1.2.5. Finally, isolated antibodies are concentrated to 5 mg/mL protein by vacuum ultrafiltration and dialyzed against PBS or 0.15 mol/L NaCl according to subsequent use.

The purity of isolated antibodies is controlled by immunoelectrophoresis and by analytical PAA gel electrophoresis; the principles are described in Chapter 1.1. Occasionally, traces of IgM antibodies can be detected. These are readily eliminated if necessary from antibodies of the IgG class by Sephadex G-200 gel filtration. For the preparation of Fab frag-

ments, i.e. the smallest available molecules from antibodies which still bind with antigen, the purified IgG-type antibodies can be finally treated with papain [86].

References

1. M. Heidelberger and F. E. Kendall, "A quantitative study of the precipitin reaction between type III Pneumococcus polysaccharide and purified homologous antibody", *J. Exp. Med.* **50** (1929) 809.
2. M. Heidelberger and F. E. Kendall, "A quantitative theory of the precipitin reaction. III. The reaction between crystalline egg albumin and its homologous antibody", *J. Exp. Med.* **62** (1935) 697.
3. S. A. Berson and R. S. Yalow, "Quantitative aspects of the reaction between insulin and insulin-binding antibody", *J. Clin. Invest.* **38** (1959) 1996.
4. E. Engvall and P. Perlmann, "Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G", *Immunochemistry* **8** (1971) 871.
5. A. H. W. M. Schuurs and B. K. van Weemen, "Enzyme-immunoassay", *Clin. Chim. Acta* **81** (1977) 1.
6. E. A. Kabat and M. M. Mayer: *Experimental Immunochemistry.* Charles C. Thomas, Springfield 1971.
7. C. A. Williams and M. W. Chase: *Methods in Immunology and Immunochemistry,* vol. 1 – 5. Academic Press, New York-London 1967 – 1977.
8. D. M. Weir: *Handbook of Experimental Immunology,* vol. 1 – 3. Blackwell, London 1978.
9. T. S. Work and E. Work: *Laboratory Techniques in Biochemistry and Molecular Biology,* vol. 1 – 9. North-Holland Publ. Co., Amsterdam-London 1969.
10. A. H. Gordon, B. Keil, K. Sebesta, O. Knessl and F. Sorm, "Electrophoresis of proteins in agar jelly", *Coll. Czech. Chem. Comm.* **15** (1950) 1.
11. S. Raymond and L. Weintraub, "Acrylamide gel as a supporting medium for zone electrophoresis", *Science* **130** (1959) 711.
12. A. L. Shapiro, E. Viñuela and J. V. Maizel, "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels", *Biochem. Biophys. Res. Comm.* **28** (1967) 815.
13. J. Oudin, "Méthode d'analyse immunochimique par précipitation spécifique en milieu gélifié", *C. R. Acad. Sci. (Paris)* **222** (1946) 115.
14. J. Oudin, "La diffusion d'un antigène dans une colonne de gel contenant les anticorps précipitants homologues. Etude quantitative des trois principales variables", *C. R. Acad. Sci. (Paris)* **228** (1949) 1890.
15. Ö. Ouchterlony, "Antigen-antibody reactions in gels", *Acta Path.* **26** (1949) 507.
16. G. Mancini, A. O. Carbonara and J. F. Heremans, "Immunochemical quantitation of antigens by single radial immunodiffusion", *Immunochemistry* **2** (1965) 235.
17. P. Grabar and C. A. Williams, "Méthode permettant l'étude conjuguée des propriétés électrophorétiques d'un mélange de protéines", *Biochim. Biophys. Acta* **10** (1953) 193.
18. C. B. Laurell, "Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies", *Anal. Biochem.* **15** (1966) 45.
19. C. B. Laurell, "Antigen-antibody crossed electrophoresis", *Anal. Biochem.* **10** (1965) 358.
20. H. G. M. Clarke and T. Freeman, "Quantitative immunoelectrophoresis of human serum proteins", *Clin. Sci.* **35** (1968) 403.
21. D. J. Gocke and C. Howe, "Rapid detection of Australia antigen by counterimmunoelectrophoresis", *J. Immunol.* **104** (1970) 1031.
22. A. Tiselius, "Electrophoresis of serum globulin. II. Electrophoretic analysis of normal and immune sera", *Biochem. J.* **31** (1937) 1464.
23. J. Uriel: "The characterization reactions of the protein constituents following electrophoresis or immuno-electrophoresis in agar". In: P. Grabar and P. Burtin (eds.): *Immuno-Electrophoretic Analysis.* Elsevier Publ. Co., Amsterdam-London-New York 1964.
24. L. Ornstein: "Discussion". In: B. J. Davis, L. Ornstein, P. Taleporos and S. Koulish, "Simultaneous preservation of intracellular morphology and enzymatic or antigenic activities in frozen tissues for high resolution histochemistry", *J. Histochem. Cytochem.* **7** (1959) 291.

25. H. Ott, "Elektrophorese in Acrylamid-Gel", *Prot. Biol. Fluids* **10** (1963) 305.
26. H. Logemann, H. Bartl., J. Nógrádi, K. Kopetz and O. Glosauer: "Allgemeines zur Polymerisation von Vinyl- und Divinylverbindungen in heterogener Phase (Emulsions-, Perl-, Suspensions- und Fällungspolymerisation)". In: E. Müller (ed.): *Methoden der organischen Chemie,* vol. XIV part 1. G. Thieme Verlag, Stuttgart 1961.
27. L. Ornstein, "Disc electrophoresis. I. Background and theory", *Ann. N. Y. Acad. Sci.* **121** (1964) 321.
28. B. J. Davis, "Disc electrophoresis. II. Method and application to human serum proteins", *Ann. N. Y. Acad. Sci.* **121** (1964) 404.
29. K. Weber and M. Osborn, "The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis", *J. Biol. Chem.* **244** (1969) 4406.
30. R. Pitt-Rivers and F. S. A. Impiobato, "The binding of sodium dodecyl sulphate to various proteins", *Biochem. J.* **109** (1968) 825.
31. G. G. Slater, "Electrophoresis on a density gradient of polyacrylamide gel. Apparatus and examples", *Fed. Proc.* **24** (1965) 225.
32. J. J. Pratt and W. G. Dangerfield, "Polyacrylamide gels of increasing concentration gradient for the electrophoresis of lipoproteins", *Clin. Chim. Acta* **23** (1969) 189.
33. J. Margolis and C. W. Wrigley, "Improvement of pore gradient electrophoresis by increasing the degree of cross-linking at high acrylamide concentrations", *J. Chromatogr.* **106** (1975) 204.
34. P. G. Righetti and J. W. Drysdale, "Isoelectric focusing in gels", *J. Chromatogr.* **98** (1974) 271.
35. R. C. Allen and H. R. Maurer: *Electrophoresis and isoelectric focusing in polyacrylamide gel.* Walter de Gruyter, New York 1974.
36. C. R. Merril, D. Goldman and M. L. Van Keuren, "Simplified silver protein detection and image enhancement methods in polyacrylamide gels", *Electrophoresis* **3** (1982) 17.
37. K. Burridge: "Direct identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels". In: V. Ginsburg (ed.): *Methods in Enzymology,* vol. 50. Academic Press, New York 1978.
38. H. Glossmann and F. Lutz, "Molecular weights of pig liver cell membrane proteins", *Hoppe-Seyler's Z. Physiol. Chem.* **351** (1970) 1583.
39. U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T 4", *Nature (Lond.)* **227** (1970) 680.
40. R. C. Allen, D. J. Moore and R. H. Dilworth, "New rapid electrophoresis procedure employing pulsed power in gradient gels at a continuous pH: the effect of various discontinuous buffer systems on esterase zymograms", *J. Histochem. Cytochem.* **17** (1969) 189.
41. A. H. Wardi and G. A. Michos, "Alcian blue staining of glycoproteins in acrylamide disc electrophoresis", *Anal. Biochem.* **49** (1972) 607.
42. E. Alpert and R. L. Coston, "Serum alpha-fetoprotein (AFP) in benign and malignant gastrointestinal diseases: evaluation of an immunoenzymatic assay", *Clin. Chim. Acta* **58** (1975) 77.
43. W. D. Kuhlmann, "Localization of alpha$_1$-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats", *Int. J. Cancer* **21** (1978) 368.
44. M. Florkin and E. H. Stotz: *Comprehensive Biochemistry,* vol. 1–8. Elsevier Publ. Co., Amsterdam-London-New York 1962.
45. R. A. Gibbons: "Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins". In: A. Gottschalk (ed.): *Glycoproteins: Their Composition, Structure and Function.* Elsevier Publ. Co., Amsterdam-London-New York 1972.
46. M. Mantle and A. Allen, "A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain", *Biochem. Soc. Trans.* **6** (1978) 607.
47. E. A. Peterson and H. A. Sober, "Chromatography of proteins. I. Cellulose ion-exchange adsorbents", *J. Am. Chem. Soc.* **78** (1956) 751.
48. R. V. Tomlinson and G. M. Tener, "The effect of urea, formamide, and glycols on the secondary binding forces in the ion-exchange chromatography of polynucleotides on DEAE-cellulose", *Biochem.* **2** (1963) 697.
49. E. A. Peterson and H. A. Sober: "Column chromatography of proteins: substituted celluloses". In: S. P. Colowick and N. O. Kaplan (eds.): *Methods in Enzymology,* vol. 5. Academic Press, New York 1962.
50. J. Porath and P. Flodin, "Gel filtration: a method for desalting and group separation", *Nature (Lond.)* **183** (1959) 1657.

51. J. Porath, "Some recently developed fractionation procedures and their application to peptide and protein hormones", *J. Appl. Chem.* 6 (1963) 233.
52. T. C. Laurent and J. Killander, "A theory of gel filtration and its experimental verification", *J. Chromatogr.* 14 (1964) 317.
53. H. Determann: *Gel Chromatography.* Springer-Verlag, Berlin-Heidelberg-New York 1969.
54. L. A. AE. Sluyterman and O. Elgersma, "Chromatofocusing: isoelectric focusing on ion-exchange columns. I. General principles", *J. Chromatogr.* 150 (1978) 17.
55. L. A. AE. Sluyterman and J. Wijdenes, "Chromatofocusing: isoelectric focusing on ion-exchange columns. II. Experimental verification", *J. Chromatogr.* 150 (1978) 31.
56. S. Hjertén, S. Jerstedt and A. Tiselius, "Apparatus for large-scale preparative polyacrylamide gel electrophoresis", *Anal. Biochem.* 27 (1969) 108.
57. A. H. Gordon: "Electrophoresis of proteins in polyacrylamide and starch gels". In: T. S. Work and E. Work (eds.): *Laboratory Techniques in Biochemistry and Molecular Biology,* vol. 1. North-Holland Publ. Co., Amsterdam-London 1969.
58. H. R. Maurer: *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis.* Walter de Gruyter, Berlin-New York 1971.
59. W. D. Kuhlmann, "Purification of mouse alpha$_1$-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization", *Histochemistry* 44 (1975) 155.
60. W. Rapp, M. Windisch, P. Peschke and K. Wurster, "Purification of human intestinal goblet cell antigen (GOA), its immunohistological demonstration in the intestine and in mucus producing gastrointestinal adenocarcinomas", *Virchows Arch. (Pathol. Anat.)* 382 (1979) 163.
61. W. Rapp and H. E. Lehmann, "Isolierung von Komponenten menschlicher Magenschleimhaut-Pepsinogene und -Cathepsine mit präparativer Polyacrylamidgelelektrophorese zur Herstellung spezifischer Immunseren", *J. Clin. Chem. Biochem.* 14 (1976) 569.
62. E. Starkenstein, "Über Fermentwirkung und deren Beeinflussung durch Neutralsalze", *Biochem. Z.* 24 (1910) 210.
63. R. Axen, J. Porath and S. Ernback, "Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides", *Nature (Lond.)* 214 (1967) 1302.
64. W. H. Scouten: *Affinity Chromatography. Bioselective adsorption on inert matrices.* J. Wiley & Sons, New York-Chichester-Brisbane-Toronto 1981.
65. P. Cuatrecasas, M. Wilchek and C. B. Anfinsen, "Selective enzyme purification by affinity chromatography", *Proc. Natl. Acad. Sci. (USA)* 61 (1968) 636.
66. J. W. Eveleigh and D. E. Levy, "Immunochemical characteristics and preparative application of agarose-based immunosorbents", *J. Solid-Phase Biochem.* 2 (1977) 45.
67. T. Kristiansen: "Matrix-bound antigens and antibodies". In: O. Hoffman-Ostenhof (ed.): *Affinity Chromatography.* Pergamon Press, Oxford 1978.
68. P. Ehrlich, "On immunity with special reference to cell life", *Proc. Roy. Soc. (Lond.)* 66 (1900) 424.
69. L. Pauling, "A theory of the structure and process of formation of antibodies", *J. Am. Chem. Soc.* 62 (1940) 2643.
70. F. Breinl and F. Haurowitz, "Chemische Untersuchung des Präzipitates aus Hämoglobin und Anti-Hämoglobin-Serum und Bemerkungen über die Natur der Antikörper", *Hoppe-Seyler's Z. Physiol. Chemie* 192 (1930) 45.
71. F. M. Burnet: *The clonal selection theory of acquired immunity.* Vanderbilt Univ. Press, Nashville 1959.
72. F. M. Burnet: *Cellular Immunology,* vol. 1 – 2. Melbourne Univ. Press and Cambridge Univ. Press, Melbourne-Cambridge 1969.
73. G. J. V. Nossal and G. L. Ada: *Antigens, Lymphoid cells and the Immune Response.* Academic Press, New York-London 1971.
74. S. De Petris and G. Karlsbad, "Localization of antibodies by electron microscopy in developing antibody-producing cells", *J. Cell Biol.* 26 (1965) 759.
75. E. H. Leduc, S. Avrameas and M. Bouteille, "Ultrastructural localization of antibody in differentiating plasma cells", *J. Exp. Med.* 127 (1968) 109.
76. W. D. Kuhlmann and S. Avrameas, "Cellular differentiation and antibody localization during the primary immune response in peroxidase stimulated lymph nodes of rat", *Cell. Immunol.* 4 (1972) 425.

77. N. K. Jerne and A. A. Nordin, "Plaque formation in agar by single antibody-producing cells", *Science* **140** (1963) 405.
78. K. Hummeler, T. N. Harris, N. Tomassini, M. Hechtel and M. B. Farber, "Electron microscopic observations on antibody-producing cells in lymph and blood", *J. Exp. Med.* **124** (1966) 255.
79. F. G. Gudat, T. N. Harris, S. Harris and K. Hummeler, "Studies on antibody-producing cells. I. Ultrastructure of 19S and 7S antibody-producing cells", *J. Exp. Med.* **132** (1970) 448.
80. W. D. Kuhlmann, "Ultrastructural immunoperoxidase cytochemistry", *Prog. Histochem. Cytochem.* **10/1** (1977) 1.
81. W. Straus, "Cytochemical observations on the development of antibody-forming cells in popliteal lymph nodes by an improved immunoperoxidase method", *Histochemistry* **53** (1977) 273.
82. C. L. Hornick and F. Karush, "Antibody affinity. III. The role of multivalence", *Immunochemistry* **9** (1972) 325.
83. E. A. Kabat: *Structural Concepts in Immunology and Immunochemistry.* Holt, Rinehart and Winston, New York 1976.
84. G. Köhler and C. Milstein, "Continuous cultures of fused cells secreting antibody of predefined specificity", *Nature (Lond.)* **256** (1975) 495.
85. A. Nisonoff, F. C. Wissler and D. L. Woernley, "Mechanism of formation of univalent fragments of rabbit antibody", *Biochem. Biophys. Res. Comm.* **1** (1959) 318.
86. R. R. Porter, "The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain", *Biochem. J.* **73** (1959) 119.
87. R. C. Valentine and N. M. Green, "Electron microscopy of an antibody-hapten complex", *J. Mol. Biol.* **27** (1967) 615.
88. G. M. Edelman and W. E. Gall, "The antibody problem", *Ann. Rev. Biochem.* **38** (1969) 415.
89. F. W. Putnam, "Immunoglobulin structure: variability and homology", *Science* **163** (1969) 633.
90. G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser and M. J. Waxdal, "The covalent structure of an entire γ-G immunoglobulin molecule", *Proc. Natl. Acad. Sci. (USA)* **63** (1969) 78.
91. H. N. Eisen: "Immunology". In: B. D. Davis, R. Dulbecco, H. N. Eisen and H. S. Ginsberg (eds.): *Microbiology. Including Immunology and Molecular Genetics.* Harper and Row Publ., Philadelphia 1980.
92. J. Clausen: "Immunochemical techniques for the identification and estimation of macromolecules". In: T. S. Work and E. Work (eds.): *Laboratory Techniques in Biochemistry and Molecular Biology.* North-Holland Publ. Co., Amsterdam-London 1969.
93. W. D. Kuhlmann, S. Avrameas and T. Ternynck, "A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates", *J. Immunol. Meth.* **5** (1974) 33.
94. O. N. Campbell, I. Luescher and L. S. Lerman, "Immunologic absorbents. I. Isolation of antibody by means of a cellulose-protein antigen", *Proc. Natl. Acad. Sci. (USA)* **37** (1951) 575.
95. I. H. Silman and E. Katchalski, "Water-insoluble derivatives of enzymes, antigens and antibodies", *Ann. Rev. Biochem.* **35** (1966) 873.
96. S. Avrameas and T. Ternynck, "The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents", *Immunochemistry* **6** (1969) 53.

2 *Preparation of Immunohistological Reagents*

2.1 Marker Molecules in Light and Electron Microscopy

The introduction of fluorescein labeled antibodies was the first breakthrough for specific investigations of cell-associated components in the light microscope [1, 2]. In the meantime, immunofluorescent procedures have reached a high standard and are successfully employed in routine immunopathology and immunohistology as well as in basic research [3]. Despite the usefulness of fluorescein and rhodamine dyes one would like to possess alternative methods for both light and electron microscopic studies. Now, a variety of labels are available. Moreover, some of them can be applied for labeling studies at the electron microscopic level.

Immuno-electron microscopy dates back to the early forties when antigen-antibody reactions were observed for the first time in an electron microscope [4, 5]. In these experiments, mixtures of virus particles and corresponding immune sera were deposited on grids, and even if the techniques employed were not as sophisticated as those used today, they were the precursors of modern dispersive immuno-electron microscopy. In principle, the resolution of the electron microscope enables the demonstration of an antibody molecule which has reacted with its antigen. Yet, unlabeled antibodies are suitable for the demonstration and characterization of isolated particles only when measurable and reproducible changes in density or definite structural changes are obtained [6, 7].

Ultrastructural identification and characterization of whole tissue *in situ* are done in transmission electron microscopes with ultrathin sections either from resin embedded organs or with non-embedded ultrathin frozen sections. In both cases, single antigenic molecules are not usually identified because such molecules have the same electron density as those of the surrounding matrix. In order to distinguish cellular antigens the employed antibodies must be characteristically "labeled" so that the resulting antigen-antibody complex becomes visible. Substances which lead to significant deflection of electrons in the electron microscope and also remain stable under the various steps of immunocytochemical organ preparation are suitable for labeling purposes. We distinguish three types of marker substances: (1) primary electron dense molecules (e.g. ferritin, heavy metals); (2) particulate substances which can be detected by their size and shape (e.g. plant viruses); (3) molecules which become electron

dense by chemical conversion (e.g. enzymes). Thus, antibody conjugates with *direct* and *indirect* visible markers can be prepared for cytological assays. In the case of enzyme-labeling, the conjugates can be also employed for light microscopic studies.

The first successfully applied labeling procedure for immuno-electron microscopic work was initiated in 1959, when Singer [8] was able to link the metalloprotein ferritin covalently to immunoglobulin fractions. Ferritin is prepared from horse spleen and contains approx. 20 % iron within its protein shell. Ferritin is a spherical molecule with a diameter of about 110 Å. The good electron scattering power conferred by the large iron content make individual ferritin molecules readily visible in the electron microscope. Even today, ferritin is still very useful as a label. Another iron-containing marker molecule, Imposil, is also commercially available (Imposil, iron-dextran prepared by Fisons Ltd., England, with 10 % (w/v) iron [9]) which can be used for the preparation of an electrondense antibody conjugate and, furthermore, which is suitable for double staining experiments concomitantly with ferritin labeled antibodies [10].

Other staining techniques were later introduced with heavy metal-labeled antibodies [11] which were based on direct labeling of antibodies with heavy metals. In subsequent years, such labeling methods were improved [12]. Many metals chelate with antibodies. However, in contrast to ferritin, the metal (e.g. uranium) must be bound in large quantities in order to obtain sufficient contrast. During labeling, uranium may also react with the specific antigen binding sites of the antibody so that the antibody should be protected during conjugation. This can be achieved by reacting the antibody with an immunoadsorbent prior to conjugation. For technical reasons the heavy metal-labeled antibody method has not found widespread applications.

Morphologically distinct compounds such as plant viruses (e.g. southern bean mosaic virus) were also proposed for the tag of cellular ligands [13]. This labeling procedure is useful for the detection of cell surface constituents, but the large marker size make it unsuitable for intracellular labeling purposes. Other direct visible markers are hemocyanin [14] being useful for ultrastructural studies of cell surface ligands and latex particles for scanning electron microscopy [15]. The disadvantage of these marker molecules is their large size. Nevertheless, plant viruses, hemocyanin and latex particles can be successfully employed in scanning electron microscopy.

In 1971, Faulk and Taylor [16] proposed colloidal gold to label immune sera. Recently, this principle was applied to protein A (from Staphylococcus aureus) for the indirect staining of antigens (Chapter 3) in ultrathin sections from resin embedded tissue [17].

Indirect marker techniques for the detection of ligands in light and electron microscopy consist of labeling immunoreactants with radioactive molecules. However, the resolution of autoradiography limits this method. More importantly, the introduction of antibodies which are covalently conjugated with enzymes proved to be a unique step forward in immunohistology [18 – 20]. Enzymes can be used which produce, upon action on their substrates, colored, insoluble and also electron dense products. Such markers make the immunohistological method very sensitive because of the amplifying ability of enzyme molecules. An enzyme is not consumed upon action with its substrate so that the final product can accumulate at the antigen site. Enzyme-labeled antibodies have the advantage over otherwise conjugated antibodies in that the same preparation can be utilized for both light and electron microscopic studies.

Many enzymo-cytochemical techniques were developed for histochemical studies [21], but only few enzymes (see later) and few histochemical methods can be reliably employed for immunocytochemical purposes. Other enzymes like myoglobin, catalase, microperoxidase may be used as tracer molecules for permeability studies. Such molecules either do not conjugate readily with antibodies or uncontrolled conjugation occurs. Finally, most of those enzymes do not possess sufficient catalytic activity.

Enzymes for immunohistochemistry should be stable (e.g. pH and temperature during conjugation, storage, incubation). Substantial amounts of the enzyme activity must be retained after conjugation, and enzymes with high specific activity and turnover numbers are preferred since with reduced activity the formation of the reaction product may be too slow to remain at the enzyme site (in the tissue) and this will cause diffusion artifacts. Finally, commercially available enzymes are of great interest because large quantities are necessary for coupling with antibodies. For adequate penetration into tissues and intracellular spaces (see Chapter 6.1), it is obvious that enzymes of low molecular weight are preferred.

Kraehenbuhl et al. [22] proposed a heme octapeptide with peroxidase activity which was prepared from cytochrome c by pepsin and trypsin hydrolysis. Then, this heme octapeptide could be conjugated with antibodies. In our opinion, the main disadvantage of such heme octapeptide conjugates is that the enzymatic activity is considerably less than that of horseradish peroxidase after its conjugation with antibody. Hitherto, heme octapeptide has rarely been used in immunohistology.

From our own and other experiments with e.g., *horseradish* peroxidase, *E. coli* alkaline phosphatase and *Aspergillus niger* glucose oxidase as marker enzymes (see references [23, 24]) we deduce that especially horseradish peroxidase (EC 1.11.1.7) is an ideal enzyme for immunohistological studies. For labeling with antibodies, highly purified peroxidase preparations from horseradish (HRP) with RZ 3 [25] are employed: Reinheitszahl RZ = $A_{403\,nm}$: $A_{275\,nm}$. These preparations are highly active, approx. 250 units/mg, measured with H_2O_2 and guaiacol [26].

Enzyme-labeled antibodies are being used increasingly for diagnostic (histopathology) and research works at both light and electron microscopic levels. Moreover, simultaneous localization of several tissue antigens in the same section is possible when substrates are applied that develop reaction products of different colors [27]. In addition, double labeling experiments are also possible by combined immunohistology and autoradiography. For example, antigenic sites and incorporation of isotopically traced precursor molecules may be studied in biological structures by use of the same specimen.

Apart from the use of enzyme labeled antibodies in cytological assays, these can be also employed in gel diffusion techniques or in quantitative immuno-assays [28]. Furthermore, enzymes may be coupled to ligands other than antibodies, e.g., with lectins [29, 30], protein A [31] and avidin [32]. The latter approach is of considerable interest in quantitative enzyme immunoassays and in enzyme immunohistology. In such procedures biotin labeled antibodies and enzyme labeled avidin are used sequentially, but a variety of indirect methods are also possible [33]. In either case we can profit from the extraordinary affinity of avidin for biotin [34–36].

2.2 Coupling Reagents and Conjugation Procedures

For the conjugation of antibodies with marker molecules, reagents are needed which form stable covalent linkage. Toluene-2, 4-diisocyanate und m-xylylene diisocyanate were the first reagents to be used successfully for conjugation of antibodies with ferritin [8, 37]. Later on, other bifunctional reagents were tried for conjugation of haptens and enzymes with proteins: e.g., 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide [19], p,p'-difluoro-m,m'-dinitrophenylsulfone [38], cyanurochloride [20], tetrazotized o-dianisidine [39], 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfate [40], glutaraldehyde [41].

Coupling reagents can be divided into two major classes: (a) reagents which activate carboxyl groups to form a CO-NH bond; (b) reagents which form a bridge between amino groups. A brief summary of common classes is shown in Table 2-1. Details of the various coupling reagents and equations for some representative reactions are described elsewhere [42, 43].

Table 2-1: Coupling Reagents.

Reagents	Reaction steps	Water solubility	pH	Solvent
Carbodiimides	1	high or low[a)]	5.5	H_2O or nonaqueous[a)]
Diisocyanates	2	low	(a) 7.5 (b) 9.5	H_2O
Halonitro-benzenes	1 or 2	low	8.5 or 10.0[a)]	acetone/ H_2O
Isoxazolium salts	1 or 2	high	5.5	H_2O
Alkylchloro-formates	2	low	(a) – (b) 9.0	dioxane dioxane/H_2O
Diazonium salts	1	high	7.5	H_2O
Imidoesters	1	high	9.0	H_2O
Glutaraldehyde	1 or 2	high	(a) 6.8 (b) 9.5	H_2O

[a)] Solubility, solvent, pH depend on the various coupling reagents within the given class

While the choice of a coupling reagent is often a matter of personal preference, in certain instances there are important advantages to choosing a particular procedure. The goal of protein-protein coupling is limited by the type of functional groups available in the given molecules. Reaction of two different proteins with a bifunctional reagent can give rise to a range of products including 1:1 conjugates, polyconjugates and polymers of each of the reac-

tant proteins. Furthermore, conjugation products may be generated in which catalytic and immunological activities (enzyme and antibody, respectively) are lost due to steric factors. Hence, during conjugation we have to consider the activity of the immunological tool on the one hand and the activity of the marker substance on the other hand. Labeling reagents which react with ε-amino groups are less destructive of antibody activity than reagents like diazonium compounds reacting with tyrosine which is within or near the specific combining sites of antibodies. Also, excessive labeling must be avoided so as not to interfere with the specific binding site of the antibody molecule by steric hindrance. Last but not least, the final conjugate should be small-sized in order to facilitate its penetration into the cells (see preembedment immuno-staining, Chapter 6).

2.2.1 "One-stage" and "two-stage" reactions

Conjugation can be carried out in so-called "one-stage" and "two-stage" reactions. In the one-stage procedure, bifunctional coupling reagent is added to a mixture of two proteins to be coupled. The disadvantage of this type is that differences in the rate of reaction of the proteins with coupling reagents can lead to selective polymerization of one of the proteins. Thus, two-stage reactions are preferred. The two-stage coupling procedures can be divided into reactions which use:

(a) Hetero-bifunctional reagents, i.e. reagents containing two different reactive functional groups. Kraehenbuhl et al. [22] described an approach for coupling a heme octapeptide with Fab fragments by use of such a reagent. The single N-terminal amino group of heme octapeptide is first reacted with the N-hydroxysuccinimide ester of p-formylbenzoic acid. In the second step, the aldehyde groups of the derivatized heme octapeptide forms a Schiff base with an amino group of the Fab fragment. Then, the conjugate is treated with reducing reagent (sodium borohydride) to prevent a reversal of Schiff base formation. This conjugation procedure is quite laborious, but, more importantly, the main disadvantage of such heme octapeptide conjugates is that the enzymatic activity is considerably less than that of HRP after its conjugation with antibody.

(b) Homo-bifunctional reagents in which the reactivity of one of the functional groups is modified by steric or electronic factors as is the case in toluene-diisocyanate. In toluene-diisocyanate, the $N=C=O$ group ortho to the CH_3 is hindered and less reactive than the para group. Then, the para group combines preferentially in the first step (e.g., with ferritin), while the ortho $N=C=O$ group remains unreacted until γ-globulin is added in the second step [37]. The first conjugation step takes place under mild conditions at pH 7.5, then, the second step is performed at pH 9.5. Since the coupling reagent is insoluble in phosphate buffer, it is difficult to predict the extent of the reaction with ferritin and the amount of isocyanate available for the second step.

(c) Reagents differing in reactivity towards the two proteins to be conjugated. The conjugation of antibodies with HRP by use of glutaraldehyde was introduced by Avrameas [41]. Glutaraldehyde was earlier employed for tanning in the leather industry [44], and this dialdehyde was later introduced by Sabatini et al. [45] for the fixation of biological specimens in electron

microscopy. Fundamental ideas on the reaction of glutaraldehyde with proteins were published by Quiocho and Richards [46] and by Bowes and Cater [47]. Amino acid analyses have shown that glutaraldehyde reacts almost exclusively with ϵ-amino groups of proteins. Conjugation of HRP with antibody is preferably done in a "two-step" technique [48], and we profit from the differing reactivity of glutaraldehyde towards these two proteins. In a first step, free ϵ-amino groups of HRP react with one of the two aldehyde groups, and the configuration of the obtained molecules is such that the second free aldehyde group does not couple with another HRP molecule; thus, being available for combination with an amino group of antibody added in the second step. Since lysine residues or NH_2-terminal amino acids are not essential parts of the specific antigen binding sites of antibodies [49, 50] inactivation during conjugation is rather unlikely. This type of conjugation became possible only because of the very low proportion of lysine in HRP (about 2 %) [51]. This explains also, why HRP alone does not polymerize even in an excess of glutaraldehyde. In such experiments it was seen that only about 10 – 20 % of HRP molecules were present as dimers.

(d) Specific modifications of proteins to ensure selective reactions. Nakane and Kawaoi [52] reported a method of coupling antibodies to HRP by modification of the HRP molecules. In the first step, α- and ϵ-amino groups of HRP are blocked by treatment with fluorodinitrobenzene and then the carbohydrate portion of the enzyme is oxidized by sodium periodate to give an aldehyde. The second step is then the reaction of HRP-aldehyde with antibody, and conjugation occurs by formation of a Schiff base between aldehyde and amino groups.

Finally, so-called protected conjugation can be achieved by reacting the antibodies with an immunoadsorbent prior to conjugation with the marker molecules. Thus, the antigen binding sites of antibodies can be specifically protected during the coupling process, and loss of immunological activity is minimized.

2.2.2 Antibody-enzyme conjugation by use of glutaraldehyde

As described above, conjugation of antibodies with enzymes or with other marker molecules is preferably done in two steps. The principle of conjugation by use of glutaraldehyde is seen in Fig. 2-1. First, the marker is treated with an excess of glutaraldehyde. Then, unreacted aldehydes are eliminated by gel filtration. In the second step, the activated marker is mixed with antibody.

Apart from the use of isolated antibodies and pure markers, an essential prerequisite for reproducible results is the employment of pure glutaraldehyde. Besides the monomer, commercial glutaraldehyde often contains various amounts of polymers, α, β-unsaturated aldehydes after aldol condensations, glutaric acid and inorganic substances (see Beilsteins' Handbuch der Organischen Chemie E III 1.3111) which all together may initiate unexpected reactions. In these cases, the degree of purity of the glutaraldehyde must be improved e.g., by vacuum distillation over a Vigreux column [53, 54]; degree of purity P. I. = $E_{235\,nm} : E_{280\,nm} < 0.2$. In the following, we describe procedures for two-step conjugations of antibodies with the most common enzymes, i.e. peroxidase and glucose oxidase. The conjugation of ligands

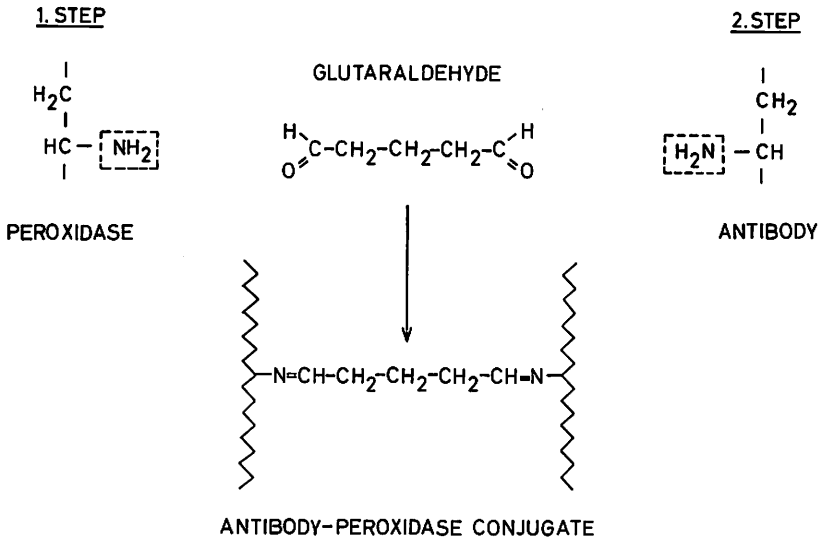


Figure 2-1. Two-step conjugation of antibodies with peroxidase by use of glutaraldehyde.

other than antibodies, e.g. lectins and protein A, with enzymes or conjugation with other markers like ferritin follows the same principle.

Procedure of conjugation of HRP (EC 1.11.1.7) with antibodies:

1. 10 mg peroxidase RZ 3 are dissolved in 0.2 mL of 0.1 mol/L phosphate buffer pH 6.8 and mixed dropwise with 0.2 mL of 2 % glutaraldehyde (in same buffer). The reaction mixture is kept for 18 h room temperature and is continuously stirred. Then, unreacted aldehydes are eliminated by Sephadex G 25 fine gel filtration equilibrated in 0.15 mol/L NaCl. The brown colored fraction contains the activated peroxidase and, if needed, is concentrated to 1 mL. A 10-fold molar excess of activated HRP is used for conjugation of antibodies.
2. 1 mL of activated HRP is mixed with 1 mL antibody (5 mg antibody per mL 0.15 mol/L NaCl) and 0.3 mL of 0.5 mol/L carbonate buffer pH 9.5 is added. The mixture is stirred at 4 °C for 24 h. The conjugate is then dialyzed against 0.2 mol/L ethanolamine-HCl buffer pH 7.4 (2 x 24 h) and, finally, against PBS (2 x 24 h) at 4 °C; sterile filtration through 0.22 μm filter, storage at 4 °C [24, 48, 55]. For long storage of purified conjugates see also last paragraph in Chapter 2.3.3.

Procedure of conjugation of glucose oxidase (EC 1.1.3.4) with antibodies:

1. Commercial glucose oxidase (GOD) is first passed on a Sephadex G 200 column, then the GOD monomer is activated with glutaraldehyde: 7.5 mg GOD in 1 ml of 0.1 mol/L phosphate buffer pH 6.8 are mixed dropwise with 0.1 mL of 1 % glutaraldehyde (same buffer). The mixture is stirred for 12 h at 4 °C. Unreacted aldehydes are eliminated by Sephadex G 25 fine gel filtration equilibrated in 0.15 mol/L NaCl, and the activated GOD is concentrated to 2 mL for conjugation with antibodies. Alternatively, the above reaction mixture may be passed on a Sephadex G 200 column in order to collect only the activated GOD monomers.
2. 2 mL of activated GOD are mixed with 1 mL antibodies (5 mg antibodies per mL 0.15 mol/L NaCl), then 0.5 mL of 0.5 mol/L carbonate buffer pH 9.5 is added. The mixture is stirred at 4 °C for 24 h. The conjugate is dialyzed against ethanolamine-HCl buffer and PBS, then concentrated and stored as described for HRP conjugates.

2.3 Purification and Characterization of Enzyme Conjugates

Because unconjugated antibodies will interfere with labeled antibodies by competing for antigenic sites, analytical applications of conjugates rely on both (a) the purity of the conjugates and, in the case of enzymes as markers, (b) its enzymatic activity. Thus, purification of conjugates is strongly recommended. Some concentration of conjugated antibodies may be achieved by ion exchange chromatography or classical salt precipitations. However, specific purification is not obtained by those techniques because unconjugated antibodies and marker molecules usually do not differ significantly in their charge and salt precipitation characteristics as compared with conjugated antibodies.

2.3.1 Purification by gel filtration

Separation of macromolecules according to their size by use of gel filtration in columns (physical separation) is widely employed in biochemistry. Thus, mixtures of conjugated antibodies, unconjugated antibodies and free marker molecules can be separated on the basis of molecular size sieving by passage through a suitable column bed of granulated gels. For the isolation of peroxidase-labeled antibodies gel filtration with Sephadex G 200 superfine may be employed. To this aim, a column (bed size: 100 x 2 cm) is packed with the gel, equilibrated with PBS or Tris-saline (0.02 mol/L Tris-HCl pH 7.2 plus 0.15 mol/L NaCl) and first calibrated with substances of known molecular weight: catalase (230000), glucose oxidase (160000), horseradish peroxidase (40000) and cytochrome c (13000). Subsequently, conjugation products are filtrated in the same column. The elution profile is shown in Fig. 2-2. The first peak contains mainly peroxidase conjugated antibodies which appear in the void volume. However, unconjugated antibodies are still detectable when the first peak is submitted to analytical PAA electrophoresis and parallel gel plates are stained for peroxidase activity and proteins. The bulk of unconjugated antibodies is obtained with the second peak; free peroxidase appears in the last one.

2.3.2 Gel filtration and lectin binding technique

Furthermore, purification of peroxidase conjugated antibodies may be obtained on the basis of immunological bindings (affinity chromatography; 1st step: insolubilized homologous antigen; 2nd step: insolubilized anti-peroxidase antibodies) or on the basis of lectin bindings. In the latter case, we employ a combination of gel filtration and Concanavalin A binding [56].

The first step is gel filtration on Sephacryl S-200 (90 x 2 cm) equilibrated with 0.05 mol/L phosphate buffer pH 7.2 plus 0.1 mol/L NaCl. Fractions of 2.5 mL are collected (Fig. 2-3) and peak I contains both labeled and unlabeled antibodies. The second step is affinity chromatography on a Sepharose 4B – Concanavalin A column (5 x 1 cm) equilibrated with 0.05 mol/L phosphate buffer pH 7.2 plus 0.1 mol/L NaCl. Peak I from the Sephacryl S-200 column is applied to the second column which is subsequently washed with excess of the start-

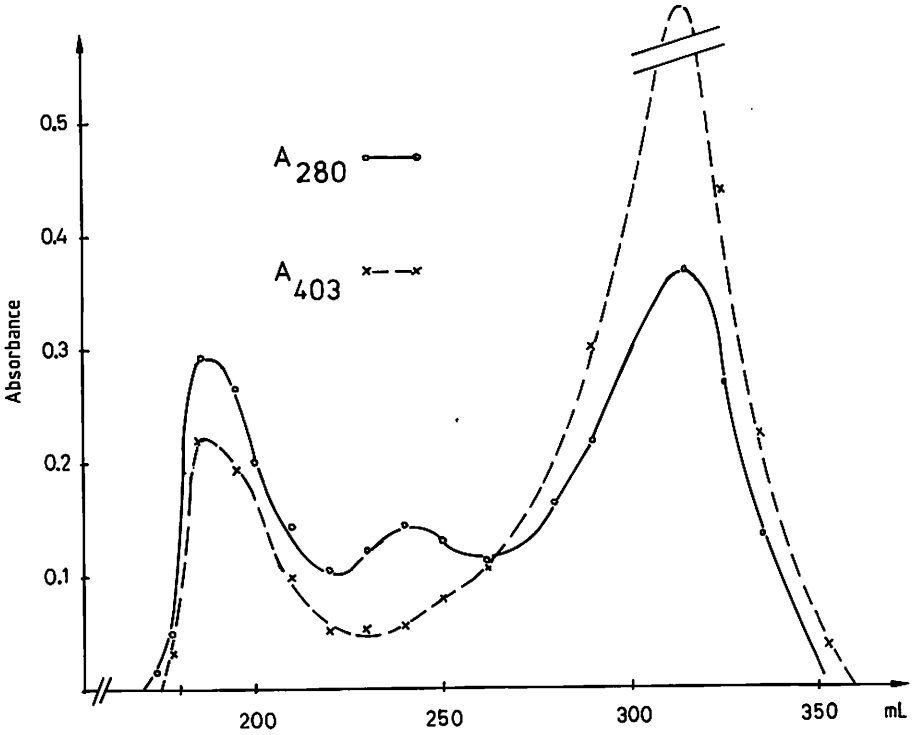


Figure 2-2. Elution profile of HRP conjugated antibodies. Individual fractions were remeasured in the spectrophotometer.

ing buffer; fractions of 1 mL are collected, flow rate 0.25 mL per minute. The column flow-through (peak A) contains the unlabeled antibodies which are not bound to Concanavalin A. Then, elution of the peroxidase-labeled antibodies (peak B) is performed with 0.01 mol/L α-methyl-D-mannoside in starting buffer (Fig. 2-3).

2.3.3 Qualitative and quantitative characterization of conjugates

After purification of conjugates their qualitative and quantitative characterization should be performed. Useful and easy procedures are immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis, immunohistological tests and quantitative immuno-assays. The easiest way to examine the conjugates is to submit them to immuno-electrophoretic analysis [57] followed by enzyme stain (see Chapter 3.2). This technique shows if antibody-enzyme conjugates still contain immunological and enzyme activities. In the following example, peroxidase-labeled anti-AFP antibodies are examined by such a method (Fig. 2-4). It can be seen that the conjugates react with AFP and stain strongly for peroxidase activity. When the conjugate itself is submitted to electrophoresis and reacted with amniotic fluid, we observe a precipitation line in the IgG region which stains for peroxidase.

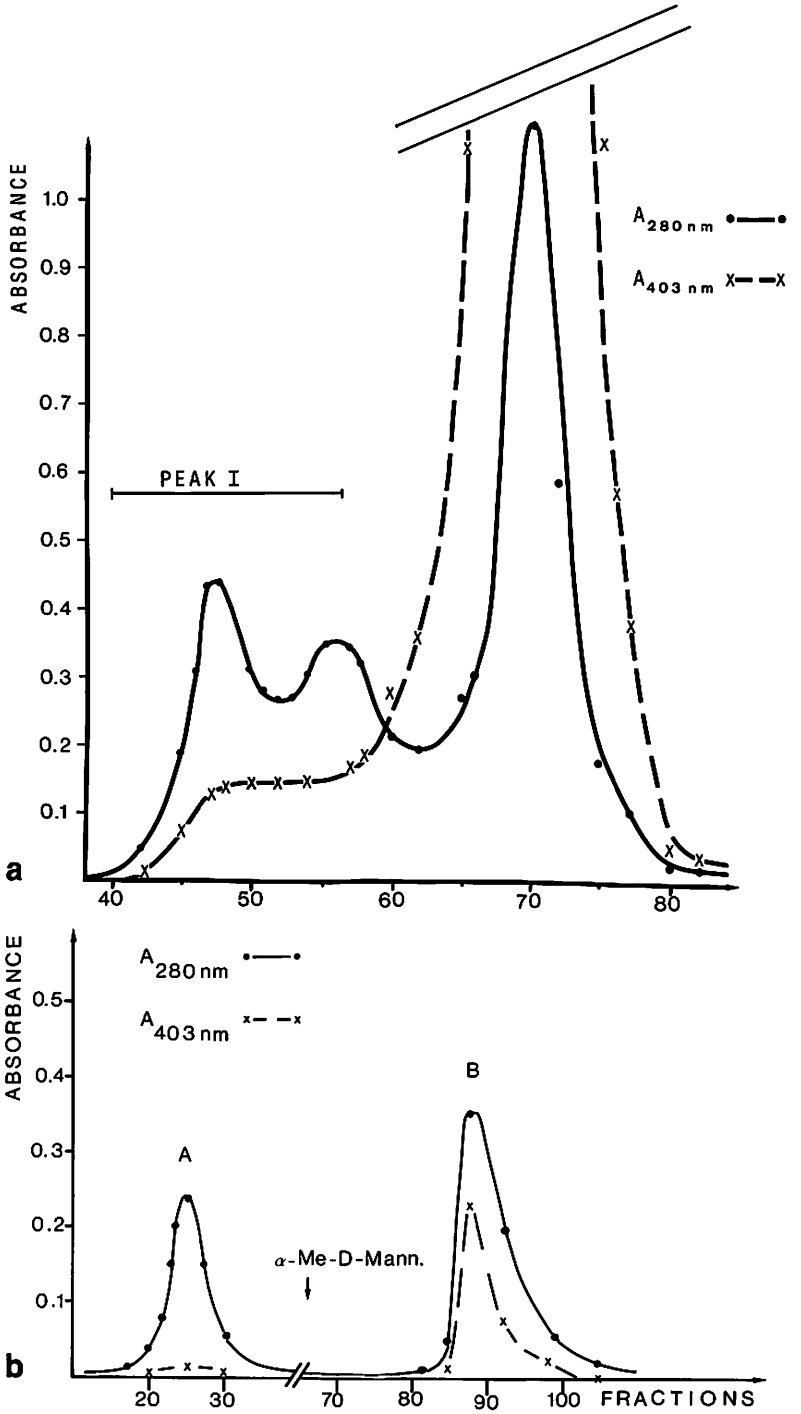


Figure 2-3. Purification of conjugates; (a) Sephacryl S-200 gel filtration; (b) Concanavalin A affinity chromatography, see text.

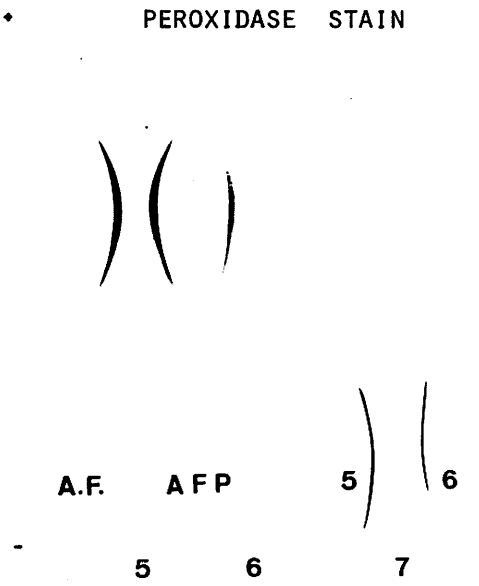


Figure 2-4. Immunoelectrophoretic analysis of HRP conjugates; rat amniotic fluid (A.F.), alpha$_1$-fetoprotein (AFP), purified anti-AFP-HRP conjugate (5), purified anti-AFP-HRP conjugate 1:32 diluted (6), rat amniotic fluid (7).

The advantage of two-stage conjugation over one-stage reactions has already been discussed in Chapter 2.2. Irrespective of the marker enzyme used, we observe in immuno-electrophoretic analysis that the heterogenous and high molecular size one-stage conjugates penetrate the gel sluggishly whereas two-stage conjugates readily reach the corresponding antigenic sites. The given example shows the situation clearly for GOD labeled antibodies (Fig. 2-5).

Figure 2-5. Immunoelectrophoretic analyses of GOD conjugates; rabbit normal serum (8) is submitted to electrophoresis and subsequently reacted with goat anti-rabbit IgG-GOD "one-step" conjugate (9) and goat anti-rabbit IgG-GOD "two-step" conjugate (10).

Apart from immuno-electrophoretic analysis, a simple possibility of examining conjugates is to stain tissue sections or cell smears which contain the corresponding antigen. Yet, it must be assured that the latter is correctly preserved for such an immunohistological assay.

Even if gel diffusion techniques and immunohistological tests can demonstrate both immunological and enzymatic activities of conjugates, all this is only semiquantitative. Quantitative data can be obtained by other immuno-assays. Thus binding studies in affinity chromatography have shown that after one-stage conjugation approximately 40 % of the 7S antibodies retained their activity. However, because of inadequate purification of one-step conjugates, these results must be regarded with caution. Then, in the case of purified two-step conjugates we found that about 50 % of the 7S antibodies and Fab fragments still react specifically with homologous antigen (Table 2-2).

Table 2-2: Determination of Immunological Binding of Conjugates and Measurement of Peroxidase Activity During Coupling Procedure.

	Specific binding with antigen	Catalytic activity of peroxidase
7 S antibody – HRP "one – step"	39.8 ± 3.2	not done
7 S antibody – HRP "two – step"	54.5 ± 3.6	69.8 ± 6.7
F ab – HRP "two – step"	51.5 ± 2.9	71.1 ± 8.1
Peroxidase RZ 3	–	100
Peroxidase RZ 3 glutarald.	–	90.5 ± 2.2

Mean values from 6 experiments, percent ± standard deviation compared with unlabeled isolated antibody and untreated peroxidase

Comparison of one-step and two-step conjugates indicated that various hindrances arose at the antigen binding sites of antibody molecules. In uncontrolled conjugation (one-step conjugation) a substantial diminution of the immunological reactivity must be reckoned with. After excess labeling of Fab fragments with HRP, Mannik and Downey [58] were also able to observe reduction of the immunological reactivity. With optimal conjugation in the two-step technique, one peroxidase molecule was coupled to one antibody molecule. The reduction in immunological reactivity is thereby kept as low as possible, though it cannot be completely avoided.

Measurements of the specific catalytic activity of horseradish peroxidases (method described in Chapter 8) have shown that under our conditions glutaraldehyde treatment only led to a maximum 10 % loss in activity. On the other hand, the loss after covalent conjugation to antibody molecules was about 40 %, and diminution of catalytic activity after conjugation of peroxidase molecules to antibodies might be due to steric hindrance of the enzyme. Nevertheless, the residual activity was sufficiently high for site-specific cytological staining.

Gel filtration techniques with calibrated columns enable molecular weight determinations. A rapid procedure for that purpose is also electrophoresis of purified conjugates in SDS-polyacrylamide gels. Thus, it was shown that homogenous conjugates can be obtained with glutaraldehyde as a bifunctional reagent used in a two step way. In such conjugates, the molar ratio of antibody or Fab to peroxidase was one. Comparable results were described elsewhere [24, 48, 55, 58].

No attempts were made with our conjugates to quantitate the amount of antibodies or Fab fragments being labeled with peroxidase. Mannik and Downey [58] reported on conjugation of Fab fragments with HRP by use of glutaraldehyde in a two-step procedure and found that about 50 % of the Fab fragments could be labeled when reacted with an 8 – 10 fold molar excess of activated peroxidase molecules.

While whole immune sera might be kept frozen or lyophilized, freezing and lyophilization of our conjugates was not very successful inasmuch as conjugates showed turbidities after thawing and reconstitution. We preferably store our conjugates in a refrigerator at 4 °C without additives. For long storage, the above prepared HRP conjugates are equilibrated with PBS by dialysis and passed through a sterile 0.22 μm filter. Stock solutions in the order of 1 mg to 2.5 mg/mL proved useful. Higher concentrations are not necessary. Such concentrations are easily achieved by use of vacuum ultrafiltration with virtually no loss of protein. No significant loss of either immunological or enzyme activities were measured within one year. Prolonged storage of diluted conjugates is not recommended because lowered protein concentration may lead to instability of the conjugates.

2.4 *Production of Soluble Peroxidase – Antiperoxidase Complexes*

Apart from the use of peroxidase labeled antibodies (covalent linkage between peroxidase and antibody), immunological bridge techniques which do not require covalent conjugation of antibody with marker may be employed in immunohistology (see Chapter 3.1). The use of soluble peroxidase-antiperoxidase complexes (PAP) is especially popular (review at Sternberger [59]).

Antibodies precipitate with corresponding antigens under certain conditions, but when antigens are in excess soluble antigen-antibody complexes will be formed. It was found that immune precipitates of HRP and anti-HRP antibodies do not dissolve readily and that in the antigen-antibody reactions between HRP and its antibody nonionic forces predominate (other forces are generally van der Waals forces and hydrogen bonding). Upon acidification of HRP-anti-HRP complexes ionic interactions become weakened, but separation of molecules will only occur when a small excess of HRP is added at the same time. Sternberger's theoretical considerations and experiments have confirmed that under such conditions added peroxidase possesses equal affinity for anti-HRP as HRP in the immune complexes and due to HRP in excess dissociation of the precipitates is achieved (to be read at Sternberger [59]); formation of soluble HRP-anti-HRP complexes (PAP) become complete and remain soluble upon neutralization.

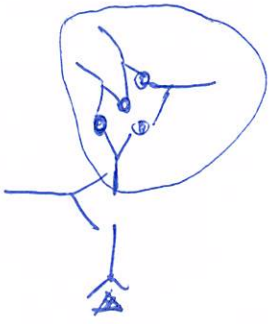
In the original preparation procedure for PAP complexes, specific immune precipitates were first obtained by mixing anti-HRP with HRP. Then, precipitates were washed in saline, resuspended by aspiration in saline containing four times the amount of HRP used for precipitation and acidified to pH 2.3. After subsequent neutralization and removal of undissolved complexes, PAP complexes were finally purified from free HRP by ammonium sulfate precipitation. Modifications have been described [60, 61] which simplify the original method but do not give significant advantage over that described by Sternberger [59, 62]. The typical protocol is given in the following paragraphs.

Protocol of PAP complex preparation:

1. The equivalence zone of rabbit anti-HRP immune serum is qualitatively determined, the AgX equivalence proportion of HRP is recorded as described in detail [59] and in Chapter 8.3.
2. PAP is preferably prepared by precipitation of anti-HRP immune serum with about 1.5 times AgX equivalence proportion. Sternberger uses 24 mg HRP for the initial precipitation in the production of PAP complexes, and he gives a typical model calculation: 24 mg HRP represents 1.5 times AgX equivalence proportion and 16 mg is the AgX equivalence proportion. When in the preliminary test (see above) the AgX equivalence proportion is recorded in the tube in which 0.4 mg HRP per mL was added to the immune serum, then a total of 40 mL of anti-HRP is needed to precipitate 16 mg HRP at the equivalence proportion or 24 mg at 1.5 times AgX equivalence proportion.
3. Preparation of PAP: HRP (RZ 3) is dissolved in 0.15 mol/L NaCl to give a final concentration of 0.4 %;

- 6 mL HRP solution (= 24 mg HRP) is mixed with 40 mL anti-HRP immune serum. After 1 h at room temperature, precipitates are collected by centrifugation for 20 min at 4 °C and resuspended (forced through pipette) in small volume of cold 0.15 mol/L NaCl, then washed by addition of 100 – 200 mL 0.15 mol/L NaCl; three washing steps are performed each followed by centrifugation;
- precipitates are carefully resuspended at room temperature in 24 mL of 0.4 % HRP made in 0.15 mol/L NaCl. The mixture is acidified under continuous stirring to pH 2.3 by addition of HCl followed by neutralization to pH 7.2. – 7.4. with NaOH; add 2.4 mL acetate solution (0.08 mol/L sodium acetate and 0.15 mol/L ammonium acetate). This mixture is cooled to 0 ° – 4 °C and centrifuged in the cold for 10 min at 15000 – 20000 rpm; the supernatant is collected. All subsequent steps are at 0 ° – 4 °C;
- the supernatant is mixed with equal amounts of chilled saturated ammonium sulfate and stirred for 30 minutes. The product is centrifuged as in the previous step and washed once with ammonium sulfate at half saturation. After centrifugation the precipitate is dissolved in 24 mL A. dest. and dialyzed extensively against three batches of large volumes of sodium ammonium acetate saline. Remaining precipitates are centrifuged as above and the supernatant is collected. PAP complexes are in the supernatant.

HRP and anti-HRP contents in soluble PAP complexes can be determined by absorbance measurements in the spectrophotometer at $A_{400\ nm}$ and $A_{280\ nm}$, respectively (see Chapter 8). The average molar ratio of HRP to anti-HRP will usually be 3:2; the yields of HRP and anti-HRP in soluble PAP complexes are about 30 %. All original steps of soluble PAP preparation and a description of its properties were described [59, 62]. It must be stated that one must expect a loss of catalytic activity of HRP being complexed in soluble PAP complexes. This loss can be quite substantial in various preparations and Bosman et al. [61] found diminutions of catalytic activity in the order of 20 – 50 % as compared with the activity of untreated HRP. Such a loss of enzyme activity may also occur in antibody-HRP conjugates after covalent linkage (see Chapter 2.3.3). For long storage, aliquots of PAP may be frozen and kept at – 20 °C.



References

1. A. H. Coons, H. J. Creech, R. N. Jones and E. Berliner, "The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody", *J. Immunol.* **45** (1942) 159.
2. A. H. Coons and M. H. Kaplan, "Localization of antigen in tissues: improvement in a method for the detection of antigen by means of fluorescent antibody", *J. Exp. Med.* **91** (1950) 1.
3. R. C. Nairn: *Fluorescent Protein Tracing.* Churchill-Livingstone, Edinburgh 1976.
4. T. F. Anderson and W. M. Stanley, "A study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum", *J. Biol. Chem.* **139** (1941) 339.
5. M. von Ardenne, H. Friedrich-Freksa and G. Schramm, "Elektronenmikroskopische Untersuchung der Präcipitinreaktion von Tabakmosaikvirus mit Kaninchenantiserum", *Arch. Ges. Virusforsch.* **2** (1941) 80.
6. J. Almeida, B. Cinader and A. Howartson, "The structure of antibody-antigen complexes. A study by electron microscopy", *J. Exp. Med.* **118** (1963) 327.
7. J. K. Blasie and M. M. Dewey, "Electron microscope and low angle x-ray diffraction studies on outer segment membranes from the retina of the frog", *J. Mol. Biol.* **14** (1965) 143.
8. S. J. Singer, "Preparation of an electron-dense antibody conjugate", *Nature (Lond.)* **183** (1959) 1523.
9. P. R. Marshall and D. Rutherford, "Physical investigations on colloidal iron-dextran complexes", *J. Colloid Interface Sci.* **37** (1971) 390.
10. A. H. Dutton, K. T. Tokuyasu and S. J. Singer, "Iron-dextran antibody conjugates: general method for simultaneous staining of two components in high-resolution immunoelectron microscopy", *Proc. Natl. Acad. Sci. (USA)* **76** (1979) 3392.
11. F. A. Pepe, "The use of specific antibody in electron microscopy. I. Preparation of mercury labelled antibody", *J. Biophys. Biochem. Cytol.* **11** (1961) 515.
12. L. A. Sternberger, E. J. Donati, J. P. Petrali, J. S. Hanker and A. M. Seligman, "Method for enhancement of electron microscopic visualization of embedded antigen by bridging osmium to uranium-antibody with thiocarbohydrazide", *J. Histochem. Cytochem.* **14** (1966) 711.
13. U. Hämmerling, T. Aoki, H. A. Wood, L. J. Old, E. A. Boyse and E. De Harven, "New visual markers of antibody for electron microscopy", *Nature (Lond.)* **223** (1969) 1158.
14. M. J. Karnovsky, E. R. Unanue and M. Leventhal, "Ligand-induced movement of lymphocyte membrane macromolecules. II. Mapping of surface moieties", *J. Exp. Med.* **136** (1972) 907.
15. A. F. LoBuglio, J. J. Rinehart and S. P. Balcerzak, "A new immunologic marker for scanning electron microscopy", *Proc. Fifth Ann. Scanning Electron Microscopy* **II** (1972) 313.
16. W. P. Faulk and G. M. Taylor, "An immunocolloid method for the electron microscope", *Immunochemistry* **8** (1971) 1081.
17. J. Roth, M. Bendayan and L. Orci, "Ultrastructural localization of intracellular antigens by the use of protein A-gold complex", *J. Histochem. Cytochem.* **26** (1978) 1074.
18. P. K. Nakane and G. B. Pierce, "Enzyme labeled antibodies. Preparation and application for the localization of antigens", *J. Histochem. Cytochem.* **14** (1966) 929.
19. J. S. Ram, P. K. Nakane, D. G. Rawlinson and G. B. Pierce, "Enzyme-labeled antibodies for ultrastructural studies", *Fed. Proc.* **25** (1966) 732.
20. S. Avrameas and G. Lespinats, "Enzymes couplées aux protéines: leur utilisation pour la détection des antigènes et des anticorps", *C. R. Acad. Sci. (Paris)* **265** (1967) 1149.
21. A. G. E. Pearse: *Histochemistry. Theoretical and Applied.* Churchill-Livingstone, Edinburgh 1972 and London 1980.
22. J. P. Kraehenbuhl, R. E. Galardy and J. D. Jamieson, "Preparation and characterization of an immuno-electron microscope tracer consisting of a hemeoctapeptide coupled to Fab", *J. Exp. Med.* **139** (1974) 208.
23. S. Avrameas, "Immunoenzyme techniques: enzymes as markers for the localization of antigens and antibodies", *Int. Rev. Cytol.* **27** (1970) 349.
24. W. D. Kuhlmann, "Ultrastructural immunoperoxidase cytrochemistry", *Prog. Histochem. Cytochem.* **10/1** (1977) 1.

25. H. Theorell and A. C. Maehly, "Untersuchungen an künstlichen Peroxydasen", *Acta Chem. Scand.* 4 (1950) 422.
26. H. U. Bergmeyer: *Methods of Enzymatic Analysis.* Verlag Chemie, Weinheim-New York 1974.
27. P. K. Nakane, "Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat", *J. Histochem. Cytochem.* 16 (1968) 557.
28. E. Engvall and P. Perlmann, "Enzyme-linked immunosorbent assay (ELISA). Quantitative assays of immunoglobulin G", *Immunochemistry* 8 (1971) 871.
29. Ch. Huet and J. Garrido, "Ultrastructural visualization of cell-coat components by means of wheat germ agglutinin", *Exp. Cell Res.* 75 (1972) 523.
30. P. J. Stoward, S. S. Spicer and R. L. Miller, "Histochemical reactivity of peanut lectin-horseradish peroxidase conjugate", *J. Histochem. Cytochem.* 28 (1980) 979.
31. M. Dubois-Dalcq, H. McFarland and D. McFarlin, "Protein A – peroxidase: a valuable tool for the localization of antigens", *J. Histochem. Cytochem.* 25 (1977) 1201.
32. J. L. Guesdon, T. Ternynck and S. Avrameas, "The use of avidin-biotin interaction in immunoenzymatic techniques", *J. Histochem. Cytochem.* 27 (1979) 1131.
33. S. M. Hsu, L. Raine and H. Fanger, "Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures", *J. Histochem. Cytochem.* 29 (1981) 577.
34. J. M. Becker and M. Wilchek, "Inactivation by avidin of biotin-modified bacteriophage", *Biochim. Biophys. Acta* 264 (1972) 165.
35. H. Heitzmann and F. M. Richards, "Use of the avidin-biotin complex for specific staining of biological membranes in electron microscopy", *Proc. Natl. Acad. Sci. (USA)* 71 (1974) 3537.
36. M. H. Heggeness and J. F. Ash, "Use of the avidin-biotin complex for the localization of actin and myosin with fluorescence microscopy", *J. Cell Biol.* 73 (1977) 783.
37. S. J. Singer and A. F. Schick, "The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin", *J. Biophys. Biochem. Cytol.* 9 (1961) 519.
38. P. K. Nakane and G. B. Pierce, "Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens", *J. Cell Biol.* 33 (1967) 307.
39. S. Avrameas, "Détection d'anticorps et d'antigènes à l'aide d'enzymes", *Bull. Soc. Chim. Biol.* 50 (1968) 1169.
40. S. Avrameas and J. Uriel, "Méthode de marquage d'antigènes et d'anticorps avec des enzymes et son application en immunodiffusion", *C. R. Acad. Sci. (Paris)* 262 (1966) 2543.
41. S. Avrameas, "Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies", *Immunochemistry* 6 (1969) 43.
42. C. W. Parker: "Nature of immunological responses and antigen-antibody interaction". In: W. D. Odell and W. H. Daughaday (eds.): *Principles of Competitive Protein-Binding assays.* Lippincott Co.. Philadelphia-Toronto 1971.
43. J. H. Kennedy, L. J. Kricka and P. Wilding, "Protein-protein coupling reactions and the applications of protein conjugates", *Clin. Chim. Acta* 70 (1976) 1.
44. L. Seligsberger and C. Sadlier, "New developments in tanning with aldehydes", *J. Am. Leather Chem. Ass.* 52 (1957) 2.
45. D. D. Sabatini, K. Bensch and R. J. Barnett, "Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation", *J. Cell Biol.* 17 (1963) 19.
46. F. A. Quiocho and F. M. Richards, "Intermolecular cross linking of a protein in the crystalline state: carboxypeptidase-A", *Proc. Natl. Acad. Sci. (USA)* 52 (1964) 833.
47. J. H. Bowes and C. W. Cater, "The reaction of glutaraldehyde with proteins and other biological materials", *J. Roy. Microsc. Soc.* 85 (1965) 193.
48. S. Avrameas and T. Ternynck, "Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration", *Immunochemistry* 8 (1971) 1179.
49. A. Nisonoff and D. Pressman, "Studies on the combining sites of anti-p-azobenzoate antibody. Loss of precipitating and binding capacities through different mechanisms of acetylation", *J. Immunol.* 83 (1959) 138.
50. D. Pressman, P. Stelos and A. Grossberg, "Retention of rabbit antibody during acetylation", *J. Immunol.* 86 (1961) 452.

51. M. H. Klapper and D. P. Hackett, "Investigations on the multiple components of commercial horseradish peroxidase", *Biochim. Biophys. Acta* **96** (1965) 272.
52. P. K. Nakane and A. Kawaoi, "Peroxidase-labeled antibody. A new method of conjugation", *J. Histochem. Cytochem.* **22** (1974) 1084.
53. H. D. Fahimi and P. Drochmans, "Essais de standardization de la fixation au glutaraldéhyde. I. Purification et détermination de la concentration du glutaraldéhyde", *J. Microscopie* **4** (1965) 725.
54. P. J. Anderson, "Purification and quantitation of glutaraldehyde and its effects on several enzyme activities in skeletal muscle", *J. Histochem. Cytochem.* **15** (1967) 652.
55. W. D. Kuhlmann, S. Avrameas and T. Ternynck, "A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates", *J. Immunol. Meth.* **5** (1974) 33.
56. M. Lannér, R. Bergquist, J. Carlsson and G. Huldt: "Purification of enzyme-labeled conjugate by affinity chromatography". In: O. Hoffmann-Ostenhof, M. Breitenbach, F. Koller, D. Kraft and O. Scheiner (eds.): *Affinity Chromatography*. Pergamon Press, Oxford 1978.
57. P. Grabar and C. A. Williams, "Méthode permettant l'étude conjuguée des propriétés électrophorétiques d'un mélange de protéines", *Biochim. Biophys. Acta* **10** (1953) 193.
58. M. Mannik and W. Downey, "Studies on the conjugation of horseradish peroxidase to Fab fragments", *J. Immunol. Meth.* **3** (1973) 233.
59. L. A. Sternberger: *Immunocytochemistry*. J. Wiley & Sons, New York 1979.
60. D. Y. Mason and R. Sammons, "Rapid preparation of peroxidase: anti-peroxidase complexes for immunocytochemical use", *J. Immunol. Meth.* **20** (1978) 317.
61. F. T. Bosman, G. Cramer-Knijnenburg and J. van Bergen Henegouw, "A simplified method for the rapid preparation of peroxidase-anti-peroxidase (PAP) complexes", *Histochemistry* **67** (1980) 243.
62. L.A. Sternberger, P. H. Hardy, J. J. Cuculis and H. G. Meyer, "The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes", *J. Histochem. Cytochem.* **18** (1970) 315.

3 *Histological Immuno-Enzyme Techniques*

3.1. Direct and Indirect Immunolocalizations

Before starting immunohistological experiments, one should have an idea about the cellular site of the tissue component under study: ligands may be localized in intracellular compartments, on or in cell surface membranes, and in extracellular spaces. Especially in preembedment immuno-electron microscopy (Chapter 6.1), the different possible localizations influence the choice of the marker and the immunohistological staining procedure.

Generally, either direct or indirect immunohistological techniques can be employed (Fig. 3-1). In the direct method, a specific antibody against the tissue antigen is prepared in a species A and coupled with a marker (e.g., peroxidase). Then, the tissue is incubated with this conjugate, and the obtained antigen-antibody complex is cytochemically stained. Indirect techniques, also termed "sandwich" methods, use in the first incubation step antibodies from species A against the tissue antigen (e.g., rabbit). Then, in the second step, antibodies are employed from a species B (e.g., sheep) which are directed against immunoglobulin of species A and conjugated with the marker. Another possibility is to make use of the protein A tech-

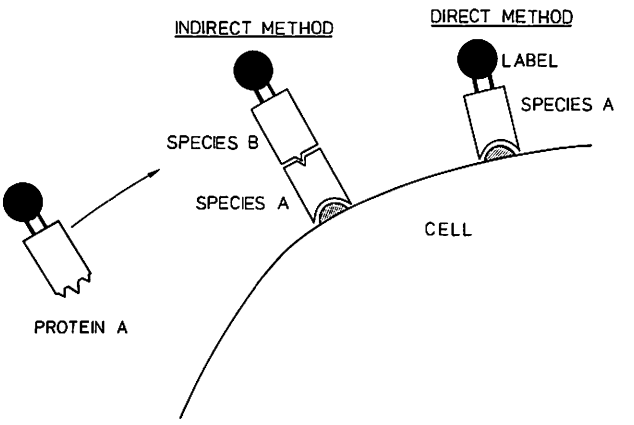


Figure 3-1. Direct and indirect immunohistological stainings, see text.

nique [1]. Protein A binds to the Fc part of the IgG, and in the example shown labeled protein A will localize the antigen-antibody complex produced by reaction of tissue antigen with its specific antibodies. Alternatively, the avidin-biotin-peroxidase technique may be employed; for technical details see Hsu et al. [2].

Other indirect localization methods involve immunological "bridging" which does not require conjugation of antibodies with a marker substance. One example is the hybrid antibody technique [3]. Hybrid antibodies contain two different antibody specificities: according to a method described by Nisonoff and Rivers [4] monovalent Fab fragments from two different IgG antibodies are combined into a bivalent hybrid molecule Fab'_2. Originally, hybrid antibodies were employed in a sandwich technique to localize cell surface antigens. In a first step, antibodies from rabbit react with cell antigen. Then, hybrid antibodies are added where one active site binds to rabbit IgG and the other active site binds to the marker which is added in a third step (Fig. 3-2); in this example, HRP is given as marker.

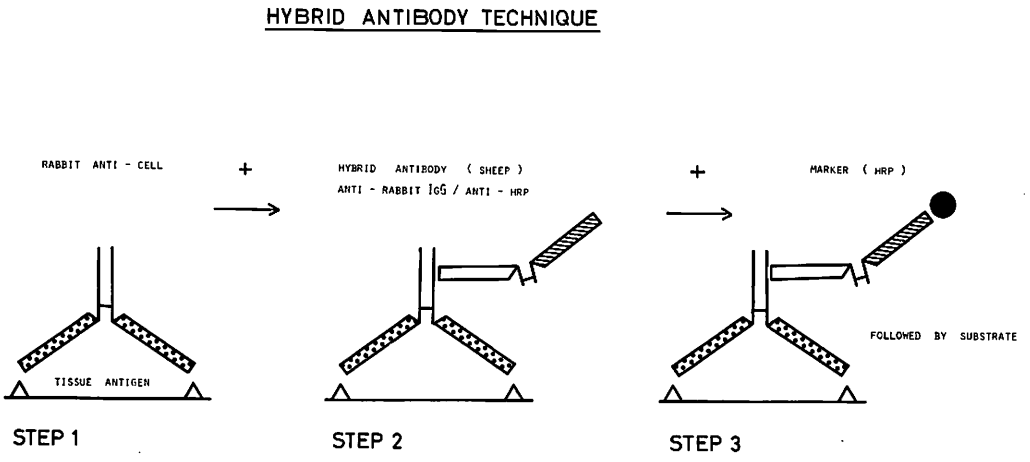


Figure 3-2. Hybrid antibody technique in immunohistology, see text.

Apart from the hybrid antibody method, numerous possibilities were proposed for immunological bridging by use of a spectrum of antibodies from different species. A popular procedure, now, is the application of soluble peroxidase anti-peroxidase complexes [5]. Cells are first incubated with antibodies (e.g., from rabbit) against tissue antigen, then with excess of sheep anti-rabbit IgG (second step). One binding site of the latter reacts with rabbit IgG of the first step, leaving free the second binding site which will bind the PAP complex added in a third incubation step (Fig. 3-3). Finally, peroxidase activities are revealed with appropriate substrate.

The choice of one of the immunohistological staining procedures is often a matter of personal preference. Direct methods are especially useful for the ultrastructural localization of antigens when the staining must be performed on tissue preparations prior to embedment and sectioning (preembedment immuno-staining, see Chapter 6.1). Otherwise, indirect procedures are useful to label components on the cell surface membranes or in extracellular spaces, or when postembedment staining can be performed on sections of embedded tissue. One example of postembedment immuno-staining is staining of sections prepared from paraffin

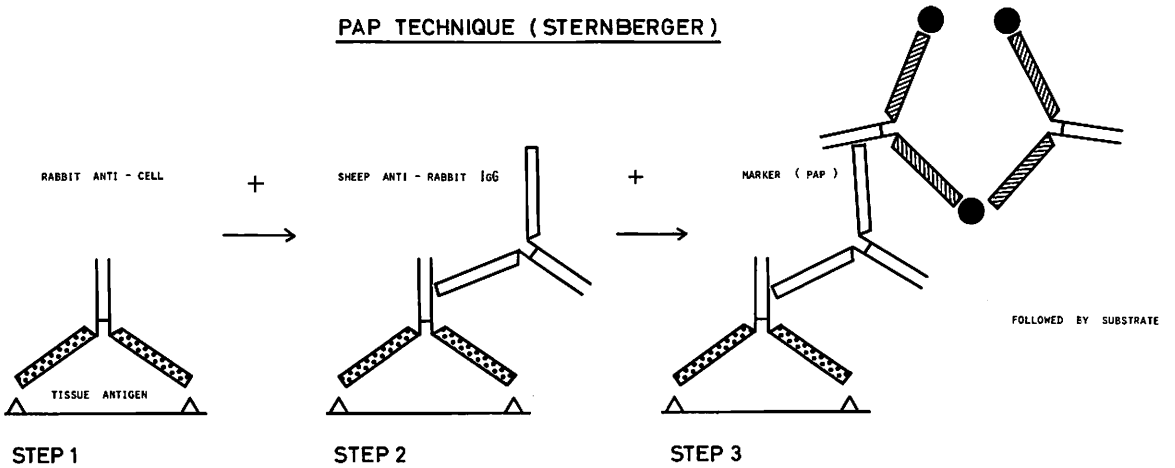


Figure 3-3. Method of Sternberger's PAP staining technique, see text.

blocks or from resin embedded material and indirect antigen localization is preferred for practical work: they make the immunohistological method very sensitive due to the amplifying effect of the sandwich antibodies. Furthermore, the latter can be employed to detect immunoglobulins of a given species irrespective of its antibody specificity.

In routine work, we follow an indirect peroxidase labeled antibody method (Fig. 3-1) in which antibodies from a species A (rabbit) directed against the tissue antigen are used in the first incubation step. Then, in the second step the formed antigen-antibody complex is tagged by HRP labeled antibodies from a species B (sheep) directed against the immunoglobulins of species A and subsequently stained by the enzyme reaction product after incubation with its substrate. The incubation time for the first serum is usually 20 min, but this step can be prolonged for at least up to 24 h (room temperature or at 4 °C) which permits the use of much higher dilutions of the first antibodies. Moreover, the specific immunohistological staining intensity is much enhanced with virtually no negative effect on non-specific background reactions when dilute solutions of the antibody are employed [6]. The localization of many different antigens was achieved with good success by this procedure and was not inferior to the PAP technique (same dilutions of primary immune sera in PAP and indirect HRP labeled antibody procedures).

Cryostat, paraffin and resin sections as well as cell suspensions, cell smears or monolayer cell cultures are incubated essentially in the same manner. Details are described in Chapters 5 and 6. In a first set of experiments, the prepared antibodies and conjugates should be submitted to chessboard titrations in order to determine their optimal concentrations. An example for such an assay is shown in Table 3-1.

3.2 *Enzyme Cytochemistry Procedures*

For the detection of peroxidase activity at light and electron microscopic levels as well as in gel plates (immuno-electrophoresis) we employ the standard staining principle developed by Graham and Karnovsky [7] (Fig. 3-4).

Table 3-1: Immunoperoxidase Localization of AFP in Paraffin Sections.

	First antibodies[a]: Isolated sheep anti-rat AFP antibodies								
	Dilutions 1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
	mg/mL 0.6	0.3	0.15	0.075	0.037	0.018	0.009	0.004	0.002
Sandwich antibodies: Anti-sheep IgG – HRP (mg/mL)									
0.24	++	++	++	++	++	++	+	(+)	Ø/(+)
0.12	++	++	++	++	++	++	+	(+)	Ø
0.06	++	++	++	++	++	+/++	+	(+)	Ø
0.03	+	+	+	+	+	+	(+)	(+)	Ø
0.015	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Ø/(+)	Ø
0.007	(+)	(+)	(+)	(+)	Ø/(+)	Ø/(+)	Ø	Ø	Ø

[a] HRP labeled antibody technique, 20 min. incubation in each antibody; immunocytochemical reactions; Ø no; (+) faint ; + positive; ++ strong positive

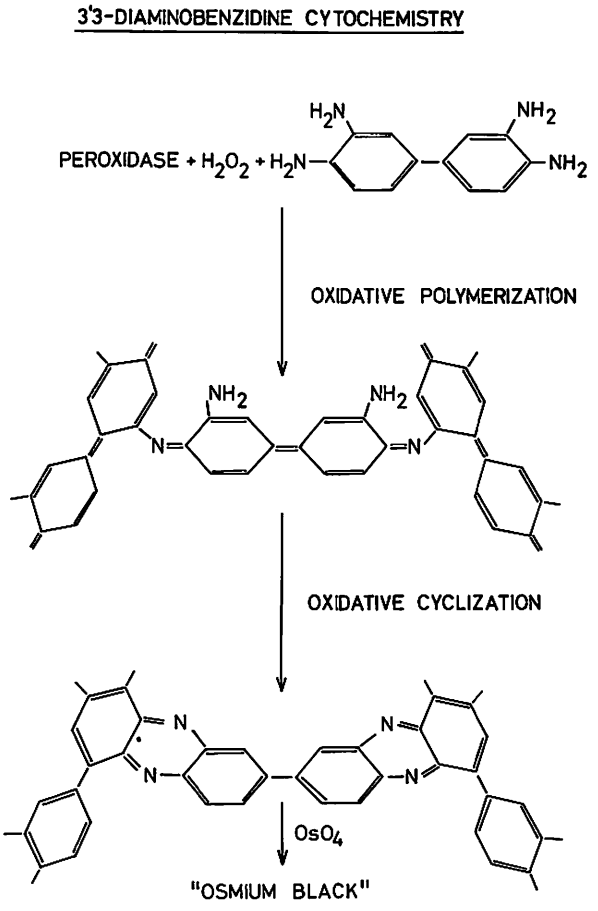


Figure 3-4. Diaminobenzidine cytochemistry, for details on substrate and final reaction product see reference [8].

Protocol of DAB cytochemistry:

1. 0.5 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB) is dissolved in 1 mL of 0.2 mol/L Tris-HCl buffer pH 7.2-7.4. Alternatively, a 10-fold stock solution of DAB may be prepared in advance and aliquots are stored at -20°C which are stable for at least one year [9]; prior to use, stock DAB is appropriately diluted. Finally, hydrogen peroxide is added to give a final concentration of 0.01 % H_2O_2, and sufficient amounts of complete substrate are employed immediately. Manipulation of the substrate needs precautions because of its potential carcinogenicity (DAB).
2. Preparations are stained for 10-20 min at laboratory temperature. Do not expose to UV light and full sun. Then, wash in several changes of PBS.
3. The final reaction product is osmiophilic, and postfixation in OsO_4 enhances the contrast in light and electron microscopies (see Chapters 5 and 6).

The reactivity of HRP can be enhanced by staining at an acid pH [10] or at a neutral pH by addition of imidazole to the DAB/H_2O_2 substrate mixture. The nitrogenous compound was found to raise the enzyme activity of HRP in test tube assays by about 30 % [11], and DAB/H_2O_2 substrate supplemented with 0.1 mol/L imidazole was successfully employed in cytochemical assays [12]. Also, the addition of cobalt chloride and nickel ammonium sulfate is reported to intensify the DAB based HRP reaction product [13].

Apart from DAB as chromogen, other enzyme substrates can be used which give different colors: e.g. 4-chloro-1-naphthol for gray-blue reaction products or 3-amino-9-ethylcarbazole for reddish reactions [14,15]. Thus, in double or triple staining experiments, antigens may be subsequently stained in the same section. The first antigen is localized by an indirect method and antibodies are removed from the section by elution (leaving the colored reaction product). Then, the second antigen is localized similarly by use of a substrate that develops a differently colored reaction product [14].

In other double labeling experiments by use of the immunoperoxidase stain and isotope tracing (e.g., detection of *in vivo* applied ^{3}H-thymidine), autoradiography is accomplished after DAB cytochemistry. For light microscopy, we employ the stripping film technique [16]. Sections are covered with Kodak AR 10 film, exposed (time is determined by trial) at 4°C and developed (e.g., Kodak D 19). Fixed and dried preparations are mounted without further staining or after hematoxylin staining in glycerol-gelatine. For high resolution autoradiography, liquid emulsion (Ilford L 4) is applied on single electron microscopic grids using the gold interference colored zone of the emulsion film in a platinum loop [17]. After exposure for several weeks/months (by trial), autoradiographs are developed by the gold latensification method [18] followed by a developer containing phenidon [19].

For the localization of glucose oxidase activity, light microscopic slides and agar gel plates as well are developed by a coupled tetrazolium salt procedure [20]:

$$\text{D-glucose} + \text{MTT}/\begin{matrix}\text{phenazine}\\\text{methosulfate}\end{matrix} \xrightarrow[(\text{FAD})]{\text{GOD}} \text{gluconate} + \text{formazan}$$

MTT = Thiazolyl blue
formazan = reduced tetrazolium salt, blue color

Protocol of GOD staining:

1. 150 mg D-glucose (anhydrous) and 10 mg MTT are dissolved in 20 mL of 0.1 mol/L phosphate buffer pH 6. 8. Then, 2 mg phenazine methosulfate are added, rapidly mixed and filtrated.
2. Microscopic slides and gel plates are immediately stained in the dark for 20-60 min (in order to avoid crystallization of MTT on the slide, add 2 mg cobalt chloride to the substrate). Slides are washed repeat-

edly in PBS (5 min) followed by distilled water and mounted in glycerol/water; gel plates are simply washed under running tap water.

For the detection of GOD activity at the electron microscopic level, a coupled enzyme system is employed [21]:

$$(1)\ \text{D-glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$(2)\ H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2\,H_2O + D$$

(DH_2 = 3,3'-diaminobenzidine tetrahydrochloride
and D = oxidized and cyclizized DAB)

Protocol of coupled enzyme staining:

1. 150 mg D-glucose (anhydrous) are dissolved in 10 mL of 0.1 mol/L phosphate buffer pH 6.8 (saturated by O_2 for 10 min). Then, 5 mg DAB are added and mixed. Finally, 1 mg HRP are dissolved in 1 mL of the above solution.
2. Preparations are stained for 20-60 min (staining solution is replaced after 30 min by a newly prepared mixture) followed by several washings in PBS.
3. Postfixation in OsO_4 can be done in order to enhance the contrast of the final reaction product.

3.3 *Control of Specificity*

The reason for many disappointing results in immunohistology is that immunocytochemical reagents are applied in a non-scientific way. The criteria for specificity are as follows:

(a) Immunohistological staining should only occur with tissue preparations which contain the appropriate antigen and must be limited to that antigen;

(b) staining should be inhibited if the conjugate is fully absorbed with homologous antigen, but not if different antigens are employed for absorption studies;

(c) no tissue staining must be observed with conjugated nonimmune IgG;

(d) staining should be inhibited by pretreatment of the tissue preparation with unconjugated antibody, the so-called blocking test.

Artefacts are either so-called false positive or so-called false negative staining reactions and depend on the prepared immunocytochemical reagents as well as on the tissue preparations. These points are major sources of artefacts:

(a) **Immunocytochemical reagents**
- antibody preparation
  (avoid contamination by unwanted and cross-reacting antibodies)

- marker substances
  (molecular heterogeneity and impurities give rise to uncontrolled conjugation)
- purification of conjugates
  (contamination by unconjugated antibodies give false negative staining results)

(b) **Tissue preparations**
- cell damage/necrosis
  (diffusion of molecules prior to fixation leads to both nonstaining areas and false positive ectopic staining in tissues)
- tissue fixation
  (false negative and false positive reactions due to diffusion by weak fixation; false negative due to denaturation of antigens)
- tissue sampling
  (denaturation of molecules by dehydration and embedment give rise to false negative staining reactions)

In practice, immunocytochemical reagents must be carefully controlled during their production (see previous chapters). Thereafter the effect of the various tissue sampling steps must be examined by combined light and electron microscopic studies. Artefacts induced by tissue preparation are considered in the following chapters. For aspects on immunohistological specificity which occur in practical work see also Chapter 7.

3.4 *Microscopy, Counterstaining and Microphotography*

Peroxidase stained preparations are observed in the light microscope with typical Köhler's bright field illumination. We usually pose a blue filter (e.g. Leitz CB 12) into the light pathway. In cases of very low contrast, the condensor lens may be slightly lowered which accentuates unstained parts of the tissue (Fig. 3-5).

Apart from contrast enhancement by postfixation with OsO_4 slides might be also counterstained with hematoxylin (Fig. 3-5) or with methyl green [22]. Photographs were taken with a commercial automatic microscope camera (24 x 36 mm) equipped with an electronic switch unit. Photos are usually recorded on 35 mm black and white negative film (e.g. Ilford Pan-F 18 DIN/50 ASA, on 35 mm tungsten color reversal film (e.g. Agfachrome 50 L, 18 DIN/50 ASA; 3 200 °K) or on daylight color reversal film (e.g. Agfachrome 50 S, 18 DIN/50 ASA; 5 500 °K). In the latter case, the lamp temperature is corrected from 3 100 °K to 5 500 °K by a conversion filter (CB 12). The exposure time is modulated by gray filter (e.g. NG 4).

Ultrastructural studies of tissues are usually performed with ultrathin sections (< 0.1 μm thick) by use of conventional transmission electron microscopes (CTEM) operating at 80 kV with a 50 μm objective aperture. In this case, sections are observed and photographed in normal bright field illumination. We used commercial film material for electron microscopy (6.5 x 9 cm; e.g. Scientia film, Agfa-Gevaert). For details on image recording see Agar et al. [23]. Sections from immuno-peroxidase reacted organs can be counterstained with lead citrate

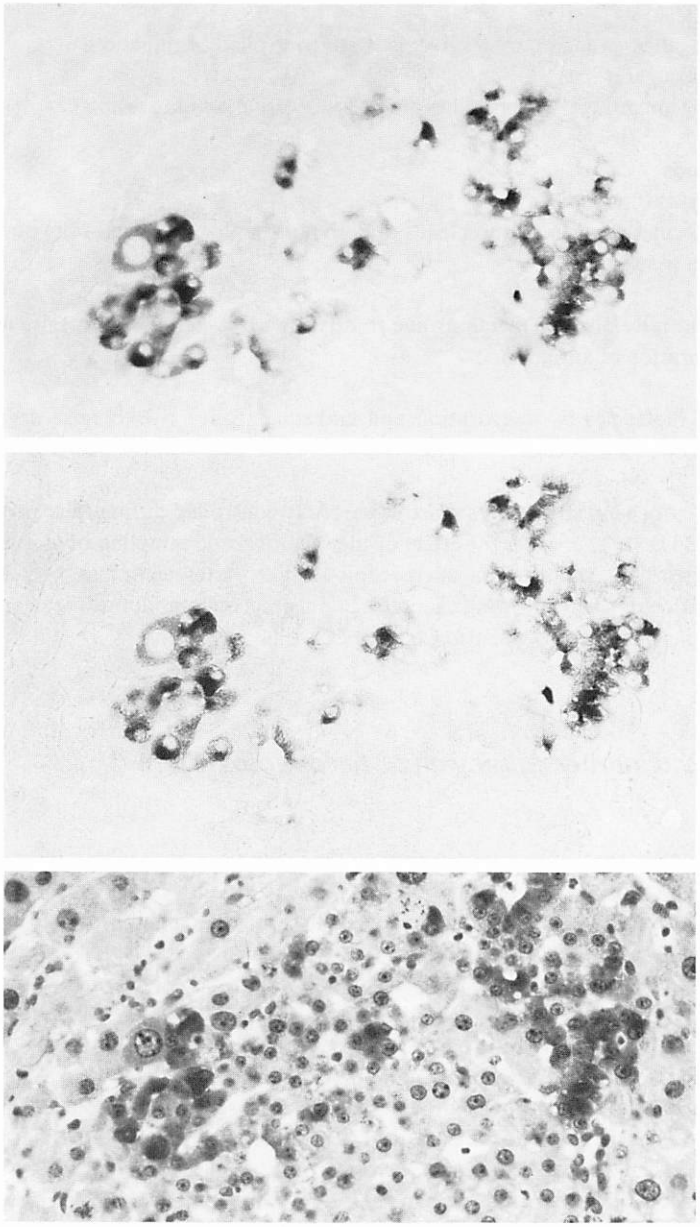


Figure 3-5. Immunoperoxidase localization of AFP in paraffin section from rat liver; experimental hepatocarcinogenesis. (a) Section is photographed in Köhler's bright field illumination; (b) same section but condensor lens slightly lowered; (c) same section but counterstained with hematoxylin. Original × 250.

[24] for a very short time (30 sec) which enhances the contrast of cells and the enzymocytochemical reactions in the section (Fig. 3-6a). Furthermore, thicker sections in the order of 1-2 μm can be evaluated in CTEM operating at 100 kV (Fig. 3-6b).For the latter purpose,

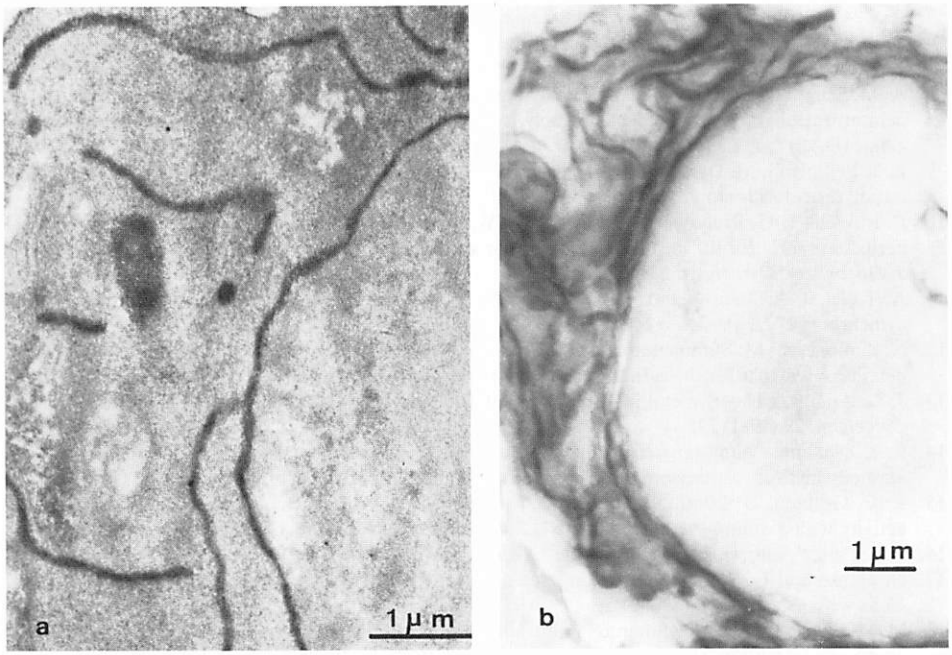


Figure 3-6. Detection of intracellular IgG by use of antibody-HRP conjugates in preembedment immunohistology; after immuno-staining, tissue was embedded in Epon. (a) Ultrathin section counterstained with lead salts for 30 sec. (b) 1 µm thick Epon section of same tissue in CTEM at 100 kV, no counterstain (from W.D. Kuhlmann, *Biol. Cellulaire* **39** [1980] 261).

however, high voltage electron microscopes with goniometer stages appear especially suitable in order to study three-dimensional organization of cells.

Distinct protocols for light and electron microscopic tissue preparations are given in the following chapters.

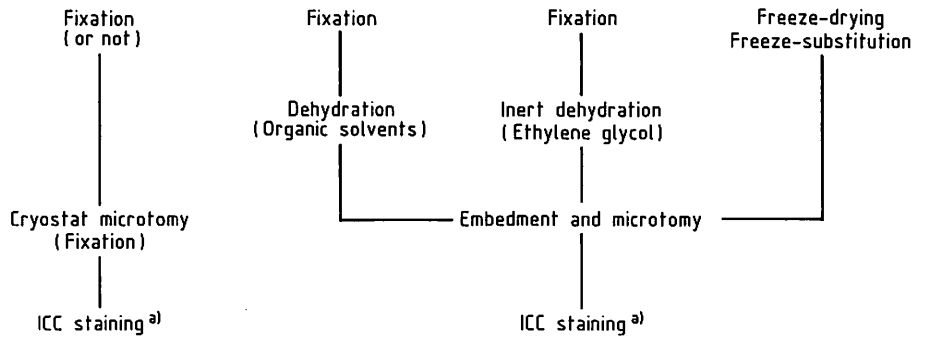
References

1. M. Dubois-Dalcq, H. McFarland and D. McFarlin, "Protein A – peroxidase: a valuable tool for the localization of antigens", *J. Histochem. Cytochem.* **25** (1977) 1201.
2. S. M. Hsu, L. Raine and H. Fanger, "Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures", *J. Histochem. Cytochem.* **29** (1981) 577.
3. U. Hämmerling, T. Aoki, E. De Harven, E. A. Boyse and L. J. Old, "Use of hybrid antibody with anti-γG and anti-ferritin specificities in locating cell surface antigens by electron microscopy", *J. Exp. Med.* **128** (1968) 1461.
4. A. Nisonoff and M. M. Rivers, "Recombination of a mixture of univalent antibody fragments of different specificity", *Arch. Biochem. Biophys.* **93** (1961) 460.
5. L. A. Sternberger: *Immunocytochemistry*. J. Wiley & Sons, New York 1979.
6. W. D. Kuhlmann, "Alpha-fetoprotein: cellular origin of a biological marker in rat liver under various experimental conditions", *Virchows Arch. (Pathol Anat.)* **393** (1981) 9.

7. R. C. Graham and M. J. Karnovsky, "The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique", *J. Histochem. Cytochem.* **14** (1966) 291.
8. A. M. Seligman, M. J. Karnovsky, H. L. Wasserkrug and J. S. Hanker, "Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB)", *J. Cell Biol.* **38** (1972) 1.
9. L. J. Pelliniemi, M. Dym and M. J. Karnovsky, "Peroxidase histochemistry using diaminobenzidine tetrahydrochloride stored as a frozen solution", *J. Histochem. Cytochem.* **28** (1980) 191.
10. E. E. Weir, T. G. Pretlow, A. Pitts and E. E. Williams, "A more sensitive and specific histochemical peroxidase stain for the localization of cellular antigen by the enzyme-antibody conjugate method", *J. Histochem. Cytochem.* **22** (1974) 1135.
11. A. T. Tu, J. A. Reinosa and Y. Y. Hsiao, "Peroxidative activity of hemepeptides from horse heart cytochrome c", *Experientia* **24** (1968) 219.
12. N. Simionescu, M. Simionescu and G. E. Palade, "Permeability of muscle capillaries to small hemepeptides. Evidence for the existence of patent transendothelial channels", *J. Cell Biol.* **64** (1975) 586.
13. J. C. Adams, "Heavy metal intensification of DAB-based HRP reaction product", *J. Histochem. Cytochem.* **29** (1981) 775.
14. P. K. Nakane, "Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat", *J. Histochem. Cytochem.* **16** (1968) 557.
15. R. C. Graham, U. Lundholm and M. J. Karnovsky, "Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole", *J. Histochem. Cytochem.* **13** (1965) 150.
16. S. R. Pelc, "Autoradiograph technique", *Nature (Lond.)* **160** (1947) 749.
17. G. Haase and G. Jung, "Herstellung von Einkornschichten aus photographischen Emulsionen", *Naturwissenschaften* **51** (1964) 404.
18. M. M. Salpeter and L. Bachmann, "Autoradiography with the electron microscope. A procedure for improving resolution, sensitivity and contrast", *J. Cell Biol.* **22** (1964) 469.
19. M. Bouteille, "Protein renewal in anti-peroxidase antibody forming cells. II. Combination of ultrastructural immuno-cytochemistry and autoradiography in "pulse-chase" experiments" *Exp. Cell Res.* **69** (1971) 135.
20. A. G. E. Pearse: *Histochemistry. Theoretical and Applied.* Churchill-Livingstone, Edinburgh 1972 and London 1980.
21. W. D. Kuhlmann and S. Avrameas, "Glucose oxidase as an antigen marker for light and electron microscopic studies", *J. Histochem. Cytochem.* **19** (1971) 361.
22. B. Romeis: *Mikroskopische Technik*. R. Oldenbourg, München-Wien 1968.
23. A. W. Agar, R. H. Alderson and D. Chescoe: "Principles and practice of electron microscope operation". In: A. M. Glauert (ed.): *Practical Methods in Electron Microscopy*. North-Holland/American Elsevier, Amsterdam-Oxford-New York 1974.
24. E. S. Reynolds, "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy", *J. Cell Biol.* **17** (1963) 208.
25. W. D. Kuhlmann, "Electron-microscopy of intracellular antigens by peroxidase labelled antibodies", *Biol. Cellulaire* **39** (1980) 261.

4 *General Preparation of Biological Specimens*

Since the introduction of histological immunolocalization of molecules a variety of tissue sampling procedures has been developed for studies at both light and electron microscopic levels [1-3]. Single cells and tissues like cellular aggregates, cell cords, solid organs or *in vitro* grown monolayer cultures are the most employed biological specimens for morphological studies. No serious difficulties are encountered when cell suspensions are submitted to immuno-staining of ligands which are located on the cell surface or in intracellular spaces. For this approach, simple procedures now being routinely used have been described extensively with immunofluorescent techniques; see references [1,4]. Briefly, cell suspensions (stabilized or not by prefixation) may be stained directly in the test tube by sequential washings and incubations with immunocytological reagents. Alternatively, cells can be smeared or centrifuged on microscope slides and are then submitted to immunostaining. Yet, a more complex situation is encountered with tissular cells (e.g., solid organs) and especially when intracellular molecules should be immuno-stained in well preserved cells. In the following, we consider the latter objective for which a general scheme is depicted:



a) See chapter 5. and 6. for light and electron microscopy, respectively

For the above purpose, distinct tissue stabilization and subsequent processing must be reconciled. Loss of immunoreactivity of antigens can occur at various stages of tissue sampling which is often a denaturation and implies irreversibility. Here, fixation, dehydration and embedment are the most important factors. We have no direct method of evaluation for the extent of conformational changes of biological substances associated with denaturation. Comparison between denaturation effects on isolated protein molecules in test tubes and those located in a cell sol (and thus sustained by other molecules) is practically impossible: phenomena of interphases, collapse, thermal movements etc. are different in both conditions and influence intramolecular forces. The latter may lead to specimen "distortion" and denaturation [5].

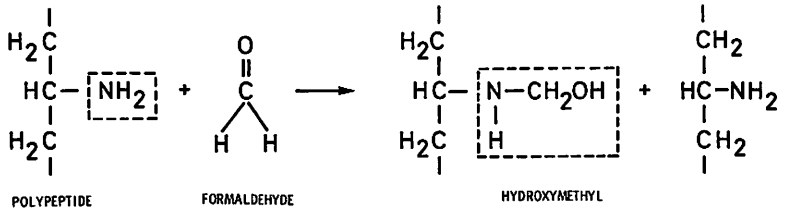
Even if the extent of all denaturation steps in tissue sampling is difficult to predict, the evaluation of sensitivity of a given specimen to its preparation can be obtained quite well by experimentation. Optimal tissue preparation depends on the type of organ and the type of substance to be detected. Because different methods will exert various actions on the specimen, we can obtain information about properties of the material under study and the usefulness of its preparation. Simple qualitative treatment will help to clarify the observed phenomena and to predict possible effects.

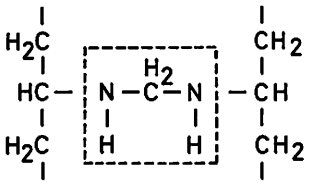
4.1 Tissue Fixation

Morphological studies usually require stabilization of cells by fixation with minimal alteration from living state and virtually no loss of tissue constituents. Tissues are composed of more and less soluble substances. For example, cell products for secretion are part of the soluble ones whereas biomolecules of the cytoskeleton are organized as a relatively insoluble structure. Hence, it is readily understood that cellular constituents not being bound to solid structures can easily lead to diffusion artefacts unless correct tissue sampling is performed.

Fixation as a process is difficult to define: on the one hand it prevents autolysis and preserves the biological structure, on the other hand it leads to protein denaturation. For most purposes, tissue fixation is done by chemical reactions. Fixatives may be organic or inorganic in nature and aldehydes and organic solvents especially are used. Fixation procedures based on water-soluble carbodiimides [6] and diethylpyrocarbonate vapors [7] have also been suggested. However, the latter as well as mixtures of periodate-lysine-paraformaldehyde [8] have not yet found widespread application in ultrastructural immunohistology.

Methanol free formaldehyde, freshly prepared from paraformaldehyde [9] and used either alone or in combination with other chemical substances, is a widely employed fixation for immunocytochemical work. Formaldehyde reacts with amino groups of proteins by formation of hydroxymethyl groups. Subsequent condensation occurs with other neighboring amino groups to form methylene bridges between polypeptide chains:

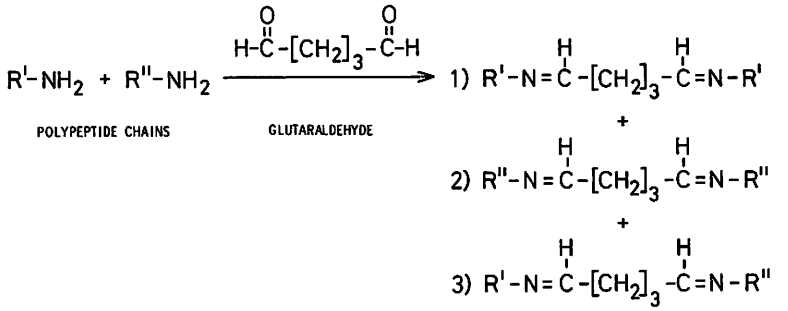




METHYLENE BRIDGE

It was found that such reactions are reversible. Boedtker [10] and Hopwood [11] have shown that formaldehyde forms only small amounts of protein polymers and that only small amounts of formaldehyde are bound by tissues during the fixation process. Thus, the question arises, what happens to the total amount of proteins, especially the soluble ones? After simple formaldehyde fixation we could observe that material was lost during subsequent immunohistological procedures [12], and, consequently, false negative findings are to be expected if the loss is significant. Furthermore, false positive staining is to be observed when insufficiently fixed material is deposited through diffusion (Chapter 7).

A more efficient fixation can be obtained with glutaraldehyde [13]. Highly purified glutaraldehyde preparations give reproducible results. The stabilizing effect must be attributed to rapid and persistent intra- and intermolecular cross-linkages of tissue components. The rate of such reactions is a factor which makes glutaraldehyde a good fixative. The degree of cross-linking is progressive with time, and depends on the accessibility of ϵ-amino groups by aldehyde groups which leads to the formation of Schiff bases:



POLYPEPTIDE CHAINS — GLUTARALDEHYDE

SCHIFF's BASE FORMATION WITH GLUTARALDEHYDE AND FREE AMINO GROUPS

Various effects of cross-linkage are obtained with proteins which possess varying amounts of available lysine groups. Two different polypeptide chains may be linked randomly to give a blend of soluble or insoluble polymers and copolymers. In the course of tissue fixation, the final outcome in cells depends on further factors like pH, ionic strength and ratio of reagent to protein.

Yet, it must be kept in mind that fixation is accompanied by an alteration of specific biological activity. The extent of denaturation, however, is difficult to predict and varies from cell to cell. Whereas the antigenicity of small peptides like hormones appear to withstand fixation quite well, proteinous antigens behave capriciously. In Fig. 4-1, parts of sperm whale myoglobin and the mode of folding of the polypeptide chain with its antigenic structure are seen. The antigenic reactive regions are few in number (shown in black), small and sharply

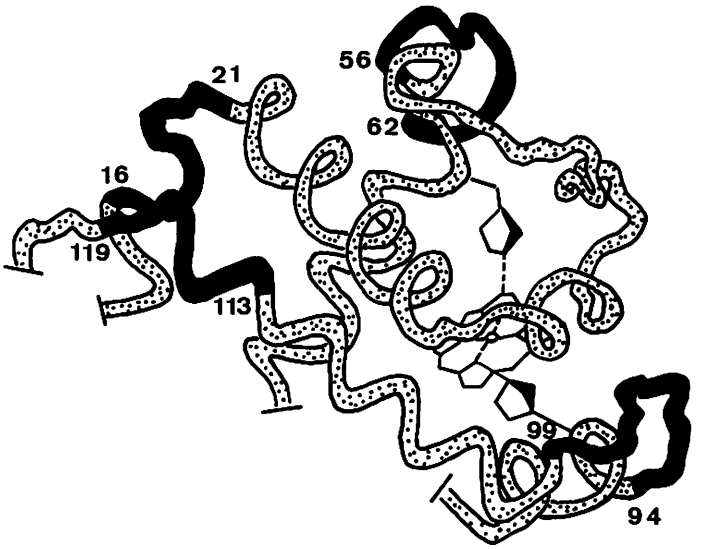


Figure 4-1. Sperm whale myoglobin (MW 17800); parts of the mode of folding the polypeptide chain and its antigenic structure (black) modified from Atassi [14].

delimited. Upon the action of aldehydes on antigenic reactive sites the immunochemical reactivity may change by modification of one/several amino groups of one/several antigenic reactive regions. At least, cross-linkages change the charge pattern of proteins and thereby already induce conformational changes. Furthermore, conformational changes outside antigenic reactive sites must be considered which also influence protein denaturation. On the one hand, aldehyde fixation can lead to masking or denaturation of antigenic determinants, on the other hand, aldehyde fixation will diminish denaturating effects by dehydration due to cross-links: sols are transformed into gels and aggregation and precipitation of proteins (collapse phenomena) are partially prevented. All such changes may occur at random.

The above considerations make it clear that fixation is a complicated and not readily controllable process. For example, molar ratios between fixative and protein molecules cannot be controlled exactly. Also, *in vitro* studies on the effect of fixatives are not directly comparable with the biological situation in situ. In our experience, the behavior of the cells during the fixation process is difficult to simulate either by gel incorporated or particle-bound proteins. So, in the course of fixation and subsequent washing processes, insolubilizations and resolubilizations may also occur in cells through the influence of complex-forming and precipitating factors which do not introduce covalent bonds. Finally, changes of cellular characteristics is practically programmed in tissue fixation and a compromise between structural conservation and retention of biological reactivity must be made. We have no evidence that the use of monoclonal antibodies will solve such problems. Yet, attempts to "reconstitute" antigenicity of protein antigens may be tried. For instance, proteolytic enzyme digestion was proposed in order to reduce background staining as well as to increase the sensitivity of specific immunohistological staining in paraffin sections [15-19].

In practice, a large variety of different fixation schedules must be examined by combined light and electron microscopy. Light microscopy will help to determine how far one can

Table 4-1: Influence of Tissue Fixation on AFP Detection.

Tissue fixation	Light microscopy (Paraffin sections) Intracellular AFP	Electron microscopy (Cryostat sections) Intracellular AFP	Cellular conservation
96 % Ethanol-1 % Acetic acid 12 – 15 h	+	–	–
4 % or 6 % Formald. 1 h	(+)	(+)[a]	not sufficient
4 % Formaldehyde 6 – 12 h	+	(+)/+[a]	not sufficient
6 % Formaldehyde 6 – 12 h	+	(+)/+[a]	not sufficient
6 % Form.-0.5 % Glut. 60 min	(+)	(+)/+	sufficient
6 % Form.-0.5 % Glut. 6 h	∅	∅	excellent
6 % Formaldehyde/5 h 6 % Form.-0.25 % Glut. 60 – 90 min	+	+	good
6 % Formaldehyde/5 h 6 % Form.-0.5 % Glut. 60 – 90 min	(+)	(+)/+	good
6 % Formaldehyde/5 h 6 % Form.-1 % Glut. 60 – 90 min	∅/(+)	∅/(+)	good

Intensity of specific reactions: ∅ no; (+) faint; + strong; – not examined
[a] Diffusion artifacts
from Kuhlmann [20]

go in tissue fixation without loss of substantial parts of immunological reactivity, whereas electron microscopy will reveal the usefulness of a given fixation schedule for ultrastructural research. Table 4-1 shows such a trial. Apart from fixation, subsequent tissue processing (dehydration, embedment) must be experimentally controlled, too.

4.2 *Tissue Dehydration*

In classical light and electron microscopy fixation is followed by dehydration. Proteins are normally suspended in the cell sap of living tissues which contain over 80 % water. The latter must be replaced by organic fluids before tissues are embedded in paraffin or poly-

merized in resins. Substitution of water will lead to various degrees of denaturation, but physico-chemical data about the fate of proteinous antigens are scarcely known. In any case, we must realize that denaturation involves both (a) loss of antigenicity for immunohistological studies and (b) loss of native structural characteristics for morphological work.

Usually low polarity solvents such as ethanol are employed for dehydration of cells. Apart from a possible extraction of tissue proteins by ethanol-water mixtures [21], a change of hydration shells of protein antigens occurs and is accompanied by denaturation. In contrast, the native conformation of proteins is less affected by the more polar ethylene glycol [22], and this agent proved to be suitable for the electron microscopic preparation of biological specimens [23-25]. Due to its two hydroxyl groups, ethylene glycol fits well into water shells of proteins so that these are not seriously disturbed and denatured.

All the above considerations must be especially taken into account for pure morphology (biophysical studies), but are not always a conditio sine qua non for immunohistology. Despite its denaturation effects, low polar solvents proved very valuable for subsequent immunohistology. During our experiments we realized that many antigens resist "denaturation" effects quite well when dehydrated with the low polar ethanol. In practice, a variety of dehydration schedules must be compared; examples for tissue dehydration are given in Table 4-2. An interesting finding was that Method A gave comparable results (e.g. postembedment immuno-staining of AFP) to those obtained by inert dehydration with ethylene glycol. However, we are aware that other antigens will behave differently. For these, alternative procedures must be elaborated.

Table 4-2: Schedules of Tissue Dehydration for Resin Embedment.

Method A	Method B	Method C
Ethanol[a]	*Ethanol*[a]	*Ethylene glycol*
30 %/30 min	70 %/30 min	10 %/30 min
50 %/30 min	95 %/2 × 30 min	gradually to 65 %
70 %/30 min	100 %/3 × 20 min	(addition of pure
90 %/60 min[b]	100 %/3 × 20 min	ethylene glycol)
95 %/30 min	prop. oxide[c]	65 %/60 min
100 %/3 × 20 min	prop. oxide/Epon	gradually to 100 %
100 %/3 × 20 min		100 %/4 × 30 min
prop. oxide[c]		prop. oxide[c]
prop. oxide/Epon		prop. oxide/Epon

[a] All steps at 0°C until 100 % ethanol was reached
[b] For Lowicryl K4M embedment see Table 4 – 3
[c] For intermediate solvents and embedment see Table 4 – 3.

4.3 *Tissue Embedment*

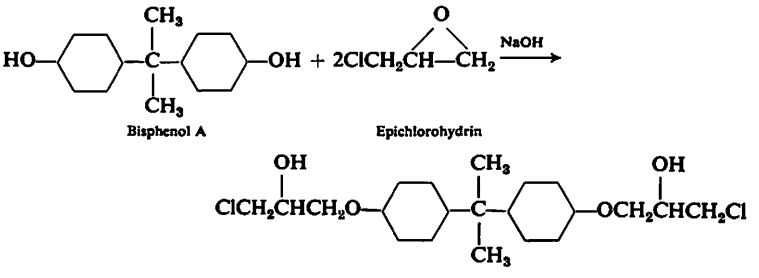
Dehydrated tissue blocks are either embedded in classical paraffin or in one of the modern resins. Paraffin embedment has proved more and more to be a reliable method for immuno-staining of intracellular antigens in sections (light microscopy) and may replace the

cryostat technique. Once tissue blocks are completely dehydrated, treatment with hot paraffin and subsequent embedment in the latter seem not to impair antigenicity. Also, low or high temperature melting paraffins have no significant influence. All our antigens tested hitherto resisted paraffin impregnation even for prolonged periods of several days in an oven at 60°C. We routinely embedded ethanol dehydrated organs via benzene or chloroform in commercial paraffin according to classical procedures [26]. Such paraffin blocks can be stored at room temperature for years without loss of immunoreactivity; postembedment immunohistology of antigens in paraffin sections see Chapter 5.1.

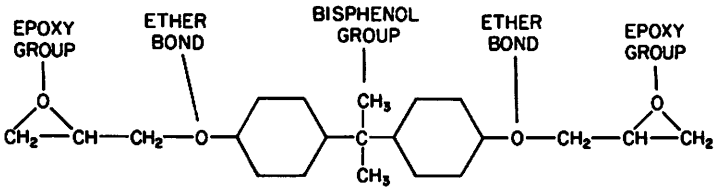
The paraffin method is still the most widely employed technique for pathohistological purposes. However, semithin sections from resin embedded organs give better morphological details. Even if immunostaining in resin sections is often associated with pitfalls, the general advantage of that method for light microscopy (semithin technique) and electron microscopy (ultrathin technique) makes this approach of special interest for the application of immunohistology in experimental cell research.

Difficulties in postembedment immunohistology are due to several factors. Loss of immunoreactivity of antigens can occur at various stages of resin embedment. Here, fixation, dehydration and final embedment are the most important points; for details on conformational changes due to fixation and dehydration see previous chapters. Further modifications and cross-linkages (inside and outside antigenic reactive sites) and both concomitant with conformational changes can occur by reaction of resin monomers or oligomers and curing agents in the final embedding step. For a better understanding, the basics of resin embedment will be briefly considered.

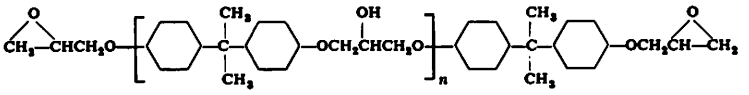
Three types of resins are usually used for electron microscopy of cells: epoxy, polyester and methacrylate resins. New, but hitherto not widely employed approaches for tissue embedment, are the use of acrylamide gels [27] and a low-temperature resin based on cross-linked acrylate-methacrylate [28]. Polyesters are also not widely employed, and methacrylates suffer from the drawback of insufficient preservation of morphology and instability in the electron beam [29]. The most employed resins are those with epoxy groups. Commercial exploitation of epoxy resins was first initiated in the mid 1930 s by I. G. Farben in Germany. Usually epoxy resins are thermosetting liquids of which the most widely used are derived from reaction between bisphenol A and epichlorohydrin in the presence of NaOH. Further reaction of the chlorohydrin intermediate with NaOH then gives diglycidyl ether of bisphenol A (DGEBA):



Chlorohydrin intermediate



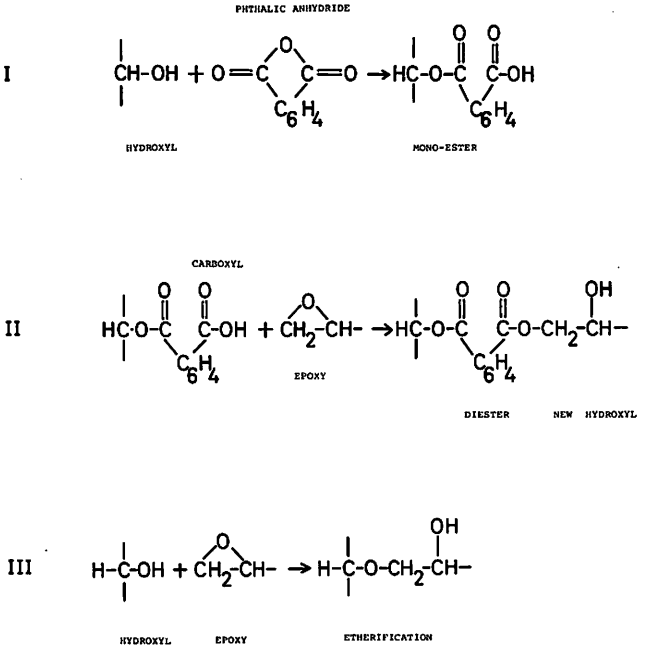
Diglycidyl ether from epichlorohydrin and bisphenol A (DGEBA)



Modified from Lee and Neville [30]

This manufacturing principle still holds true today. From the available epoxy resins, Epon® 812 (a registered trade mark by Shell) is the most common embedding material in electron microscopy. Epon 812 is a glycerol based aliphatic epoxy resin and its major components are di- and monoglycidyl but also some triglycidyl ethers of glycerol.

When epoxy monomers are cured (e.g., with phthalic anhydride), three-dimensional structures are formed by cross-links. These are principally based on di-ester bridges and ether bridges for which three reaction types are suggested [30, 31]:



In the course of Epon curing not all monomers and oligomers will react, and incompletely reacted compounds occur especially when Epon is cured stepwise with increasing temperatures [32]. In the polymerization reaction, heat is liberated over a period of time and results in an increase in the temperature (exotherm) of the reaction mixture. Exotherm profiles from curing of typical Epon 812 mixtures for electron microscopic purposes are shown in Fig. 4-2. Epon curing was done either in one step at 60 °C for 24 h or in three steps: at 35 °C for 24 h, then at 45 °C for 24 h and finally at 60 °C for 24 h [32]. Note that the exotherm reaction in the small castings (1 g) is only slightly higher than the oven temperature and is practically immeasurable when curing is done in three steps.

In view of the above observations we prefer for our immunohistological work the curing of tissues in three steps even if direct curing at 60° for 24 h also yielded sections in which immunostaining was well performed, too (see Chapter 5.3).

Recently, a polar low-temperature embedding medium was introduced for ultrastructural studies of cells, i. e. Lowicryl K4M which is based on cross-linked acrylate-methacrylate [28]. Polar media and low-temperature embedding procedures may be applied in order to reduce denaturation and conformational changes usually associated with nonpolar dehydration and resin curing [22, 25, 33]. The above K4M resin was developed for this purpose and first results including immunohistology were reported [34 – 36].

The above mentioned resins may react with amino groups of antigens and a loss of immunoreactivity will be added to that due to fixation and dehydration. The extent of such a loss is still unknown. In any event, we must expect that substantial quantities of antigen reactive sites/regions are no longer reactive. Yet, sufficient numbers can remain for subsequent postembedment immunohistology. Schedules of resin embedments with the widely used Epon 812, with a low-viscosity epoxy resin diepoxide octane (DEO), with the new polar methacrylate based Lowicryl K4M and the polyester resin Vestopal are shown in Table 4-3; for details on immunohistological results see Chapter 5.3.

4.4 *Antigen Protective Measures*

In view of denaturation effects by nonpolar dehydration and embedment, we are interested in measures which can protect antigens. One of the most important factors responsible for unsuccessful postembedment immuno-staining of tissue antigens in resin sections is their modification in the course of resin curing. Thus, attempts were made to protect protein amino groups by their reversible modification with carbobenzoxychloride (originally introduced in peptide synthesis by Bergmann and Zervas [37]) in the hope that those masked antigenic determinants could be preserved during embedding and could be restored afterwards in the tissue sections prior to immuno-staining. It was found with cryostat sections that antigens might be totally masked in a reversible manner and that nonspecific protease treatment could restore them [38], but subsequent studies have also shown that protease treatment alone was capable of restoration of great parts of protein antigens in sections of styrene-methacrylate embedded organs. Thus, it was deduced that protection of antigens by carbobenzoxychloride was not essential [39]. However, these authors reported staining difficulties with other resin embedments, where protease treatment of sections from Epon embedded

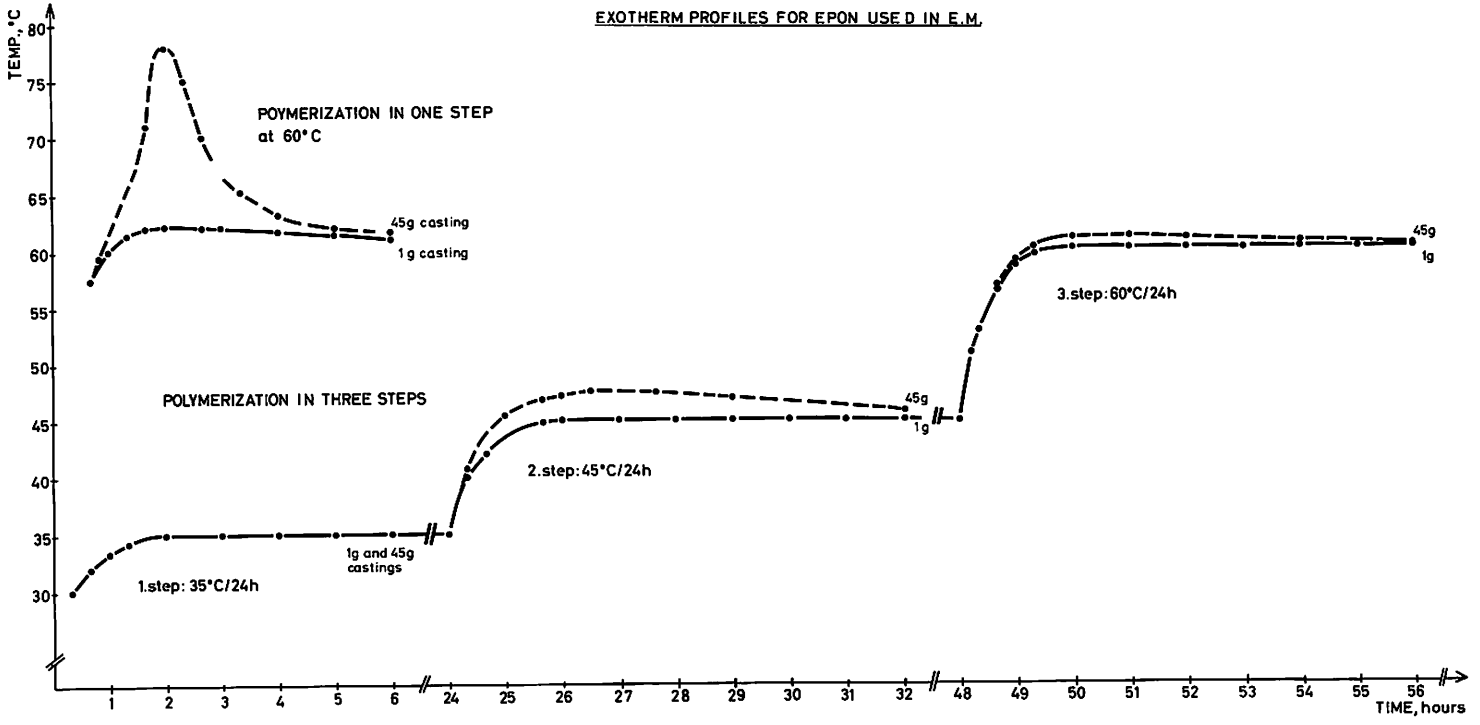


Figure 4-2. Exotherm profiles in the course of Epon curing.

Table 4-3: Formulas of Resin Embedments.

Embedding media		Intermediate solvents	Embedment polymerization
Resin	hardener accelerator		
Epon 812 87 mL (A:E = 0.70)	DDSA 90.0 mL MNA 23.0 mL DMP-30 3.0 mL	prop. oxide 2 × 30 min followed by prop. oxide/Epon ratio 1:1/60 min	(a) 35 °C/24 h 45 °C/24 h 60 °C/24 h (b) 60 °C/24 h
DEO 72 mL (A:E = 0.60)	NSA 128.0 mL DMP-30 2.6 mL	prop. oxide 2 × 30 min followed by prop. oxide/DEO ratio 1:1/60 min	(a) 35 °C/24 h 45 °C/24 h 60 °C/24 h (b) 60 °C/24 h
Lowicryl K4M monomer 260 g	cross-linker 40.0 g initiator 1.5 g	K4M/90 % ethanol ratio 1:1/60 min followed by K4M/90 % ethanol ratio 2:1/60 min followed by pure K4M overnight[a]	indirect U.V. (360 nm) 12 h at 0 °C[a]
Vestopal Vestopal W 165 g Vestopal H 35 g	BME 1.2 g	acetone 4 × 20 min followed by acetone/Vestopal ratio 3:1/30 min ratio 1:1/30 min ratio 1:3/30 min pure Vestopal over- night	indirect U.V. (360 nm) 24 h at 0 °C

Abbreviations: dodecenyl succinic anhydride (DDSA); methyl nadic anhydride (MNA); nonenyl succinic anhydride (NSA); 2, 4, 6-tri (dimethylaminomethyl) phenol (DMP-30); propylene oxide (prop. oxide); benzoin methyl ether (BME);

[a] Antigen preservation may be better following dehydration and polymerization at -30 °C [35].

tissue gave very inconsistent results inasmuch as only very weak specific staining could be obtained in a few experiments. Hence, the proposed antigen protective measure and the unmasking experiment of antigens with protease are not reliable under all circumstances. Despite such unsuccessful experiments and in view of the importance of postembedment immuno-staining it is necessary to elaborate alternative methods. Further experiments with tissue conditioning by inert compounds (filler) and suitable chemical modification of antigens are needed.

One approach tested by us was the use of polyvinylpyrrolidones (PVP, Fig. 4-3).These inert products of pharmaceutical relevance were developed by Reppe as blood plasma expander and are today used for many other purposes (review at Reppe [40]).

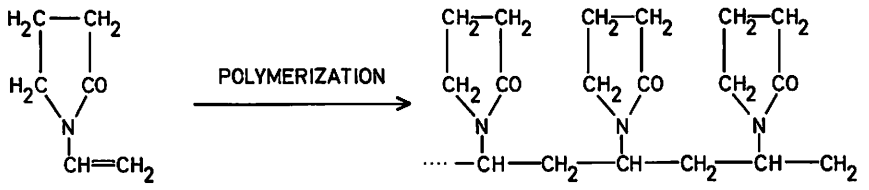
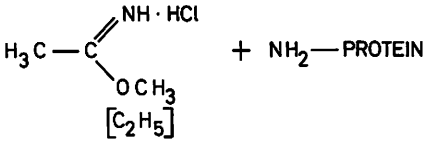


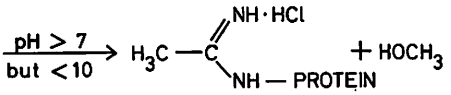
Figure 4-3. Monomeric vinylpyrrolidone and polyvinylpyrrolidone; for synthesis and structural details see Reppe [40].

PVP molecules are able to form micelles by adsorption, so that protein stabilization can be achieved. Addition of PVP to wash buffer (e.g. after aldehyde fixation), ethanol and also to Epon (via ethanol/Epon mixture as intermediate prior to embedment) was tried with two objectives: (a) antigen protection by micellar distribution of PVP; (b) incorporation of PVP in tissue as ready-for-use wash-off filler material [36].

Micellar distribution of PVP may help antigens to remain nearer to their native states. On the one hand, dehydration would have less deleterious effects on the conformation. On the other hand, antigenic determinants would be protected from reactive resin and copolymerization would be prevented to a large extent. Finally, inert filler material might be readily washed off from sections after partial removal of resin in order to enhance the penetration of immunohistochemical reagents to reach antigenic sites. All such effects of PVP are difficult to measure quantitatively, but can be assessed from experiments. Indeed, tissue conditioning with 5 or 10 % PVP improved immunoreactivity of various examined antigens.

In another approach we used the imidoester ethyl acetimidate (EAI) for reversal chemical modification of proteins [41] in order to protect antigenically reactive regions [36]. This monofunctional imidate was applied after aldehyde fixation and prior to dehydration of tissues for the conversion of free amino groups into unreactive amidines:





Technical and theoretical aspects of the chemical modification of proteins were reviewed by Means and Feeney [42]. Our idea was that EAI would protect antigens inasmuch as blocked amino groups could no longer react with epoxy groups in the course of resin curing. Thus, antigenic reactive sites would be more readily exposed upon partial removal of resin in sections and further removal of acetimidyl groups by ammonia-acetic acid pH > 11.

There is no question that dehydration and embedment of organs with low-polarity compounds will alter the immunoreactivity of tissue antigens, but excessive impairment can be reduced by appropriate schedules. Alternatively, an inert dehydration e.g. with ethylene glycol may be employed. PVP, EAI and further not yet examined treatments of fixed tissues prior to dehydration enable antigen protection in the course of dehydration and resin embedment. However, the protective effect will probably vary from one antigen to another. For some experimental data see Chapter 5.3.

References

1. R. C. Nairn: *Fluorescent Protein Tracing*. Churchill-Livingstone, Edinburgh 1976.
2. W. D. Kuhlmann, "Ultrastructural immunoperoxidase cytochemistry", *Prog. Histochem. Cytochem.* **10/1** (1977) 1.
3. L. A. Sternberger: *Immunocytochemistry*. J. Wiley & Sons, New York 1979.
4. D. M. Weir: *Handbook of Experimental Immunology*. Blackwell, London 1978.
5. E. Kellenberger and J. Kistler: "The physics of specimen preparation". In : W. Hoppe and R. Mason (eds.): *Unconventional Electron Microscopy for Molecular Structure Determination*. F. Vieweg & Sohn, Braunschweig-Wiesbaden 1979.
6. P. A. Kendall, J. M. Polak and A. G. E. Pearse, "Carbodiimide fixation for immunocytochemistry: observations on the fixation of polypeptide hormones", *Experienta* **27** (1971) 1104.
7. A. G. E. Pearse, J. M. Polak, C. Adams and P. A. Kendall, "Diethylpyrocarbonate, a vapour-phase fixative for immunofluorescence studies on polypeptide hormones", *Histochem. J.* **6** (1974) 347.
8. I. W. McLean and P. K. Nakane, "Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy", *J. Histochem. Cytochem.* **22** (1974) 1077.
9. A. Reychler, „Beiträge zur Kenntnis der Eigenschaften des Formaldehyds", *Chem. Zentralblatt* **79** (1908) 714.
10. H. Boedtker, "The reaction of ribonucleic acid with formaldehyde. I. Optical absorbance studies", *Biochem* **6** (1967) 2718.
11. D. Hopwood, "A comparison of the cross-linking abilities of glutaraldehyde, formaldehyde and α-hydroxyadipaldehyde with bovine serum albumin and casein", *Histochemie* **17** (1969) 151.
12. W. D. Kuhlmann, S. Avrameas and T. Ternynck, "A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates", *J. Immunol. Meth.* **5** (1974) 33.
13. D. D. Sabatini, K. Bensch and R. J. Barnett, "Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation", *J. Cell Biol.* **17** (1963) 19.
14. M. Z. Atassi, "Antigenic structure of myoglobin: the complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins", *Immunochemistry* **12** (1975) 423.
15. S. N. Huang, "Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections", *Lab. Invest.* **33** (1975) 88.
16. S. N. Huang, H. Minassian and J. D. More, "Application of immunofluorescent staining on paraffin sections improved by trypsin digestion", *Lab. Invest.* **35** (1976) 383.
17. H. Denk, T. Radaszkiewicz and E. Weirich, "Pronase pretreatment of tissue sections enhances sensitivity of the unlabelled antibody-enzyme (PAP) technique", *J. Immunol. Meth.* **15** (1977) 163.

18. R. C. Curran and J. Gregory, "Demonstration of immunoglobulin in cryostat and paraffin sections of human tonsil by immunofluorescence and immunoperoxidase techniques. Effects of processing on immunohistochemical performance of tissues and on the use of proteolytic enzymes to unmask antigens in sections", *J. Clin. Pathol* **31** (1978) 974.
19. T. O. Rognum, P. Brandtzaeg, H. Ørjasaeter and O. Fausa, "Immunohistochemistry of epithelial cell markers in normal and pathological colon mucosa", *Histochemistry* **67** (1980) 7.
20. W. D. Kuhlmann, "Ultrastructural detection of alpha$_1$-fetoprotein in hepatomas by use of peroxidase-labelled antibodies", *Int. J. Cancer* **22** (1978) 335.
21. J. H. Luft and R. L. Wood, "The extraction of tissue protein during and after fixation with osmium tetroxide in various buffer systems", *J. Cell Biol.* **19** (1963) 46A.
22. C. Tanford, C. E. Buckley, P. K. De and E. P. Lively, "Effect of ethylene glycol on the conformation of γ-globulin and β-lactoglobulin", *J. Biol. Chem.* **237** (1962) 1168.
23. D. C. Pease, "The preservation of unfixed cytological detail by dehydration with "inert" agents", *J. Ultrastruct. Res.* **14** (1966) 356.
24. F. S. Sjöstrand and L. Barajas, "Effect of modifications in conformation of protein molecules on structure of mitochondrial membranes", *J. Ultrastruct. Res.* **25** (1968) 121.
25. F. S. Sjöstrand and F. Kretzer, "A new freeze-drying technique applied to the analysis of the molecular structure of mitochondrial and chloroplast membranes", *J. Ultrastruct. Res.* **53** (1975) 1.
26. B. Romeis: *Mikroskopische Technik.* R. Oldenbourg, München-Wien 1968.
27. N. Yamamoto, S. Yamashita and K. Yasuda, "New embedding method for immunohistochemical studies using acrylamide gel", *Acta Histochem. Cytochem.* **13** (1980) 601.
28. E. Carlemalm, W. Villiger and J. D. Acetarin, "Advances in specimen preparation for electron microscopy. I. Novel low-temperature embedding resins and a reformulated Vestopal. *Experientia* **36** (1980) 740.
29. A. M. Glauert: "Fixation, dehydration and embedding of biological specimens". In: A. M. Glauert (ed.): *Practical Methods in Electron Microscopy.* North-Holland and American Elsevier, Amsterdam-Oxford-New York 1975.
30. H. Lee and K. Neville: *Handbook of Epoxy Resins.* McGraw-Hill, New York 1967.
31. W. Fisch, W. Hofmann and J. Koskikallio, "The curing mechnism of epoxy resins", *J. Appl. Chem.* **6** (1956) 429.
32. J. H. Luft, "Improvements in epoxy resin embedding methods", *J. Biophys. Biochem. Cytol.* **9** (1961) 409.
33. E. Kellenberger, "Progress and new approaches in obtaining finer significant details in the electron microscopy of biological material", *J. Electron Microsc.* **28** (Suppl.) (1979) 49.
34. E. Kellenberger, E. Carlemalm, W. Villiger, J. Roth and R. M. Garavito: *Low Denaturation Embedding for Electron Microscopy of Thin Sections.* Chem. Werke Lowi, Waldkraiburg 1980.
35. J. Roth, M. Bendayan, E. Carlemalm, W. Villiger and M. Garavito, "Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue", *J. Histochem. Cytochem.* **29** (1981) 663.
36. W. D. Kuhlmann and R. Krischan, "Resin embedment of organs and postembedment localization of antigens by immunoperoxidase methods", *Histochemistry* **72** (1981) 377.
37. M. Bergmann and L. Zervas, "Über ein allgemeines Verfahren der Peptid-Synthese", *Ber. Dtsch. Chem. Ges.* **65** (1932) 1192.
38. H. Takamiya, W. Bodemer and A. Vogt, "Masking of protein antigen by modification of amino groups with carbobenzoxychloride (benzyl chloroformate) and demasking by treatment with nonspecific protease", *J. Histochem. Cytochem.* **26** (1978) 914.
39. H. Takamiya, S. Batsford and A. Vogt, "An approach to postembedding staining of protein (immunoglobulin) antigen embedded in plastic: prerequisites and limitations", *J. Histochem. Cytochem.* **28** (1980) 1041.
40. W. Reppe: *Polyvinylpyrrolidon.* Verlag Chemie, Weinheim 1954.
41. M. J. Hunter and M. L. Ludwig, "The reaction of imidoesters with proteins and related small molecules", *J. Am. Chem. Soc.* **84** (1962) 3491.
42. G. E. Means and R. E. Feeney: *Chemical Modification of Proteins.* Holden-Day, San Francisco-Cambridge-London-Amsterdam 1971.

5 *Immunoperoxidase Histology in the Light Microscope*

General rules for tissue preparation as well as principles of immuno-staining and pertinent controls are considered in the above chapters. We now describe practical aspects for research and routine work. For this purpose, we demonstrate typical examples from our laboratory.

5.1 General Methods of Antigen Detection in Paraffin Sections

The localization of antigenic cell markers in histological preparations of paraffin embedded organs opens a wide field for basic research and diagnostic histopathology. From all the above can be deduced that tissue preparation needs adaption to the desired immunostaining. Yet, retrospective studies with routinely processed tissues in pathology are often possible, and this makes immunohistology a versatile technique [1]. Descriptions of some cell-associated markers and its application for histogenetic and histopathological studies of normal and diseased organs of the gastrointestinal tract are given in Chapter 5.2. First, we give standard staining protocols for the localization of antigens in paraffin sections. The material chosen is human gastric mucosa which is routinely processed for histopathological diagnostic, and the target antigen to be localized is gastrin.

Protocol of immuno-staining:

1. Tissue sampling: Usually, as described in detail in the previous chapters concerning the general concepts of immunohistology, the optimal fixation must be determined by trial (see Chapter 4. 1) followed by appropriate dehydration and paraffin embedment. In the present case, tissue blocks are fixed in classical formalin, dehydrated and embedded in paraffin as is usual in routine histopathology [2]. 5 – 7 μm thick sections are cut and mounted on acetone cleaned slides and dried at 35-40°C for 30 minutes.
2. Pretreatment: sections are passed through xylene (2 x 2 min), absolute ethanol (2 x 2 min), decreasing ethanol series (brief rinsings) into distilled water and washed in PBS (3 x 2 min). Then, preparations are passed into 1 % H_2O_2/PBS for 60 min in order to inhibit endogenous peroxidases [3]. Sections are washed again in PBS (3 x 2 min) followed by 1 % bovine serum albumin (BSA) in PBS (BSA/PBS) for 5 min; all sera are diluted in BSA/PBS. Pretreatments with H_2O_2, BSA and normal sera (from species

which provide the sandwich antibodies) improve immunohistological specificity (cf. Chapter 7 for details).

3. Incubation: either a direct or an indirect immuno-staining method may be applied. We describe here the indirect peroxidase labeled antibody method (method I) and the PAP technique (method II); sections are covered with the respective sera and incubated in a humid chamber.

(a) Method I

- sheep normal serum (sheep NS, 1: 30 diluted in BSA/PBS) for 5-10 min followed by rinsing in BSA/PBS;
- first step, rabbit anti-human gastrin (0.005-0.01 mg antibodies/mL) for 12-24 h at 4°C; in the case of whole immune sera or γ-globulin fractions, dilutions up to 1: 1000 and higher also yield good staining. Rinse and wash in BSA/PBS (3 x 5 min) at room temperature;
- second step, sheep anti-rabbit IgG labeled with HRP (0.01-0.1 mg/mL) for 20 min at room temperature. Commercial conjugates may be used in concentrations given by the manufacturer. Commercial conjugates are, however, not always safe inasmuch as uncontrollable reactions often occur (background staining). Best results are obtained with conjugates prepared according to the above specifications (Chapter 2). After incubation, sections are rinsed and washed in BSA/PBS (3 x 5 min).

(b) Method II

- sheep NS for 5-10 min followed by rinsing in BSA/PBS;
- first step, rabbit anti-human gastrin (as in Method I), optimal dilution determined by trial;
- second step, sheep anti-rabbit IgG (0.1 mg antibodies/mL or hyperimmune serum diluted 1:20 in BSA/PBS); in any case, excess of antibodies must be employed; incubation time is 20 min at room temperature, followed by washings in BSA/PBS (3 x 5 min);
- third step, PAP complex (prepared from rabbit immune serum) diluted 1:50 in BSA/PBS. PAP is commercially available. Sections are incubated for 20 min at room temperature, then rinsed and washed (3 x 5 min) with PBS.

4. Enzymo-cytochemical detection of HRP: DAB cytochemistry according to Chapter 3.2; the cytochemical reaction product is intensified by postfixation with 0.1 % OsO_4 in PBS for 1 min; the latter treatment ist not essential and can be omitted. Excess OsO_4 ist washed off with 70 % ethanol. Sections are mounted under coverglass, observed, photographed and counterstained if desired (see also Chapter 3.4).

5. Counterstaining: sections may be counterstained e.g., with Mayer's hemalum for 2 – 5 min or with 0.1 % methyl green in distilled water for 10 min [2]. Finally, sections are dehydrated in ascending series of ethanol and mounted in resinous medium under coverglass.

The result of gastrin localization in routinely processed tissue by the indirect peroxidase labeled antibody method is shown in Fig. 5-1. The above described incubation schedules also prove useful for a variety of other antigens and other organs (see Chapters 5.2 and 5.3).

As indicated previously, a prerequisite for reliable immunolocalization of antigens is the use of highly purified immunocytochemical reagents in appropriate concentration (determined by titration). Antibodies purified by affinity chromatography are preferred even if IgG fractions give specific reactions, too. So-called background staining is virtually suppressed when wash buffers are supplemented with bovine serum albumin. Otherwise, wash buffers might also be supplemented with NaCl in higher concentrations than usually employed in PBS (see Chapter 7). In our hands, the indirect peroxidase labelled antibody method works at least as specifically and sensitively as the PAP technique.

Commercially available kits are of great interest for users in routine laboratories. However, one must be aware that pitfalls can be encountered with such kits. They often lack specificity. Comparative studies by use of purchased kits and by use of standardized antibodies/immune sera (self-prepared or by a research laboratory) are necessary to prove reliability. Apart from suggested incubation schedules and enzyme substrates (provided with commercial kits) the examination of previously established schedules is also recommended.

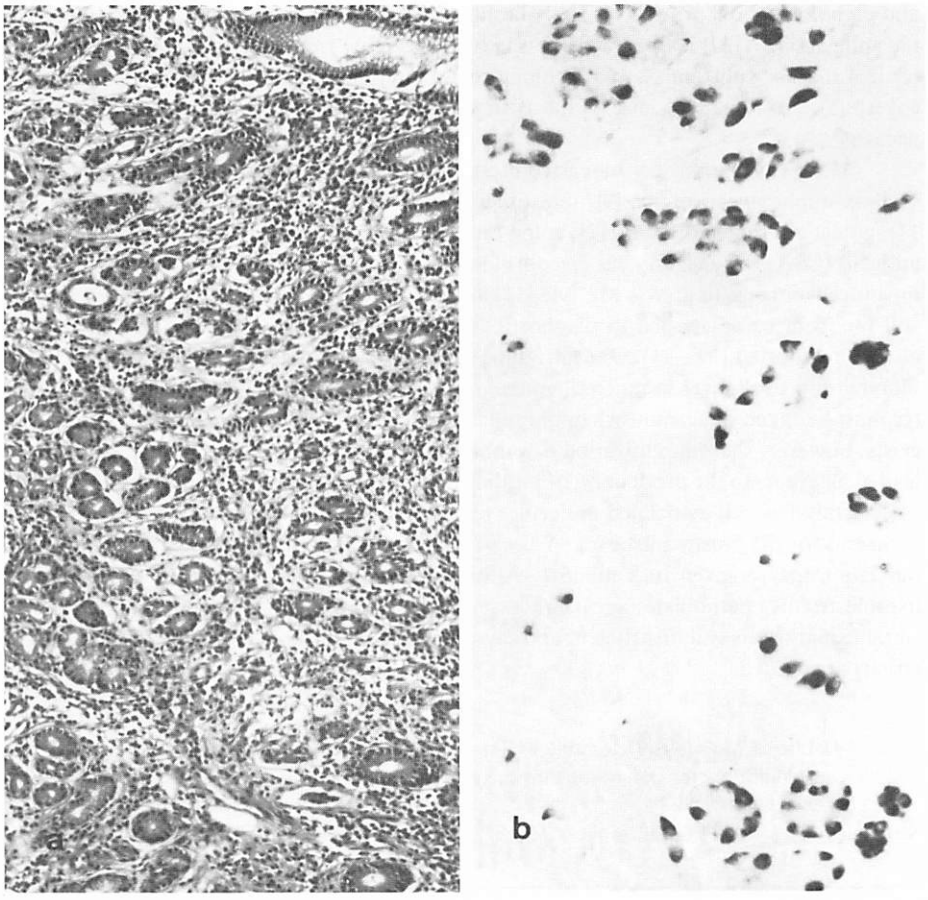


Figure 5-1. Detection of gastrin producing cells in mucosal glands. (a) H & E stain, original × 63; (b) immuno-staining for gastrin, original × 160.

5. 2 *Immunoperoxidase Localization of Cell-associated Molecules (Mucus Neutral Glycoproteins) in Gastrointestinal Mucosa*

Combined analysis of mucosal cells by biochemistry, immunology and histology provides data on their heterogeneous molecular composition. Knowledge thereof is prerequisite for the definition of cell-associated marker substances. These will enable identification of distinct mucosal cells, evaluation of cell function and elucidation of histogenetic relationships. Hence, immunohistology is one of the most important tools of functional histology and comprehensive histopathology of the gastrointestinal tract. Numerous authors have therefor used immunohistological methods in studies on mucosae.

Nairn et al. [4, 5] and De Boer et al. [6] were first in the sixties to employ immunofluorescent techniques for the localization of gastrointestinal antigens, yet knowledge of normal

and pathological cell products is fairly limited. Today, Gold and Freedman's carcinoembryonic antigen (CEA) [7] is one of the best known and most frequently studied human gastrointestinal marker substandes. Its histological distribution was described shortly after its discovery [8]. However, the lack of specificity of CEA made the search for further substances necessary.

Meanwhile, a variety of gastrointestinal antigens have been isolated and examined, such as sulphoglycoproteins [9], intestinal mucosa specific glycoproteins (GMP, IMP) [10, 11], goblet cell mucin (GCM) [12], colon mucoprotein antigen (CMA) [13, 14], colon specific antigens (CSA) [15], acid mucus glycoprotein in goblet cells (GOA) [16], gastric and intestinal mucus cell antigens (e.g. M1, M2, M3 [17] and SIMA, LIMA [18]). All of these, however, are still far from being applied in diagnostic histopathology. Special animal experiments have also been reported [19 – 21]. One intriguing factor in such studies is the natural occurrence of blood group substances in mucosal epithelium [22 – 25]. A possible interference with the latter must be taken in account when immune sera against mucosa antigens are used. Evidence exists, however, that immunization of rabbits with gastrointestinal mucosubstances does not lead at all events to the production of antibodies directed against blood group antigens[9].

Only few cell-associated molecules (e.g. enzymes, hormones) have hitherto proved to be useful for diagnostic purposes. A list of gastric marker molecules routinely employed in our laboratory is given in Table 5-1. Although immuno-staining of such substances yield useable results, pathohistological processes are as yet poorly understood. The pattern of suitable markers is still insufficient and therefor further substances of defined molecular specificity are needed.

Table 5-1: List of Molecules Detectable by Immunohistology in Human Stomach Mucosa which are Used in Our Laboratory for Diagnostic Purpose.

Molecules	Localization	Histopathological relevance	References
Pepsinogen group I and II	chief cells, mucus neck cells	atrophia, metaplasia (fundic mucosa)	[26, 27]
Pepsinogen group II	pyloric glands	atrophia, metaplasia (antral mucosa)	[27]
Esterase VI A	chief cells, mucus neck cells	atrophia, gastritis, foveolar hyperplasia	[28, 29]
Carcinoembryonic antigen (CEA)	–	dysplasia, intestinal metapl., gastric carcinoma	[30 – 32]
Gastrin	G-cells	gastrinoma, G-cell hyperplasia (antrum)	[33, 34]
Acid mucus glycoprotein (GOA)	–	intestinal metaplasia gastric carcinoma	[16]
IgA, IgM, IgG	plasma cells	gastritis, chron. inflammation, lymphoid infiltration	[35]

It is generally accepted that secretory and nonsecretory mucins play a complex role in normal physiology. Changes have been noted in their heterogeneous chemical nature during the development of benign and malignant diseases. However, the corresponding cellular processes and the exact nature of mucin changes remain unclear. One important reason being the fact that the normal composition of mucins is not well defined. Qualitative and quantitative data on the composition of mucins will most likely reveal important clues for the assessment of normal and pathological cell function and structure.

Two major types of mucins are presently distinguished by histochemical methods, i.e. acid and neutral mucins (glycoconjugates). A significant advance in the histological detection of mucins was achieved by the introduction of stainings such as periodic acid-Schiff (PAS) and Alcian blue at pH 2. 5 [36], Alcian blue at pH 1.0 [37] and high iron-diamine/Alcian blue [38] as staining agents, which chemically react with the mucins. Moreover, several special methods which induce defined chemical or enzymatic alterations in tissue sections have been developed for the staining of reactive groups in epithelial mucins [39]. However, the complexity and poor reproducibility of many of these methods has prevented their routine application. Furthermore, specificity as defined by modern biochemistry and immunology cannot be achieved. Detailed data on the complex composition of mucins, their pathways of synthesis and excretion and their cellular origin in relation to normal and abnormal development are still scanty, and the few available results remain controversal.

With these difficulties in mind we have concentrated our efforts on isolating and characterizing immunogenic glycoprotein components in neutral glycoconjugates (NGC). The methodological approach for preparation and analysis is schematically described in Chapter 1.2; a full description will be published separately in the near future. The objects studied were normal mucosae of human stomach and of human small intestine; stomach and intestine from fetal and adult rats were taken for comparison. In collaboration with my assistant Dr. Peschke we isolated several human NGC substances. A designated nomenclature for these substances will be assigned after definition of biochemical characteristics is completed. Meanwhile, different NGCs are identified by Roman numerals. At this point, we present three NGC substances with high molecular weights (around 500 000 daltons each). The different purified NGCs seem to be homogeneous inasmuch as they migrate as single-banded zones in analytical PAA gel electrophoresis. They show strong PAS reactivity much in the same way as classified neutral glycoproteins. Protein staining is weak, which indicates low protein contents. This behavior is typical for neutral glycoproteins of mucus origin. One of the NGCs (NGC I) is purified from normal human stomach, the two others (NGC II and NGC III) were obtained from human intestine. All three NGC preparations are immunogenic upon immunization of rabbits. The obtained immune sera cause specific precipitation reactions in gel diffusion techniques. These sera can be used to stain specific cell populations. For further characterization of our substances we intend to carry out biochemical, immunological and immunohistological comparisons with mucosubstances isolated in other laboratories in the near future.

During isolation and characterization of NGCs from human mucosae we observed that all obtained immune sera possessed strong crossreactivity with molecules from a variety of animal species. Such molecules normally occur in animal cells and may be considered homologues to human ones. For example, rabbit antibodies against human NGC II react with human mucus neck cells in the stomach as well as with mucus producing cells in corresponding organs of pig, rat, guinea pig and frog (other species under study). Some of the mucosal

NGCs have structural similarities and share antigenic determinants. Hence, they can be considered phylogenetically related and phylogenetically old molecules comparable to albumin or AFP. Phylogenetic and ontogenetic relationships are also evident from the observation that rabbit antibodies formed against neutral glycoproteins from rat fetal colon are capable to stain mucus neck cells in adult rat stomach as well as in adult human stomach. Such phenomena of cross-reactivity due to immunochemically related substances in different mammalian species were suggested by us earlier [40].

In the following, some immunohistological results are described with rabbit antibodies against human NGC I, NGC II and NGC III, respectively. The indirect immunoperoxidase method was applied to histological preparations from normal and diseased gastric tissues of defined blood groups*). Specimens were obtained by surgery. Detailed description of diseases such as single goblet cell metaplasia and intestinal metaplasia are found in the literature [41 – 45]. Results on gastric carcinomas which also have been studied are not included in this report. Specimens of gastric carcinomas of diffuse and intestinal types[46] were few and not sufficient to draw conclusions from the obtained immunohistological staining pattern.

Protocol of immuno-staining:

1. Tissue sampling: specimens are fixed in 99 % ethanol-1 % acetic acid for 12 – 15 h at 0 – 4°C. This fixation method proved very valuable for immunohistology even if argentaffin/enterochromaffin cells are not readily distinguished in sections stained by routine dyes. Tissue blocks are dehydrated in absolute ethanol, passed through chloroform and embedded in paraffin. 5 – 7 μm thick sections are cut and mounted on acetone cleaned slides. For routine histology, sections are stained with hematoxylin-eosin, PAS and Alcian blue at pH 2.5, respectively (see Chapter 8.6).
2. Pretreatment: for immunohistology, sections are passed through xylene, a decreasing ethanol series, distilled water and into "pretreating media" (described in Chapter 5.1) for highest possible specificity of immuno-staining.
3. Incubation: the indirect peroxidase labeled antibody method was employed (method I, Chapter 5.1); in the first step, sections are incubated for 24 h at 4°C in γ-globulin fractions of rabbit anti-human NGC I, NGC II and NGC III, respectively. In the second step, sheep anti-rabbit IgG antibodies labeled with HRP are added for 20 min at room temperature; washings between the steps are described in Chapter 5.1.
4. Enzyme-cytochemical detection of HRP: detection of enzyme activity using DAB cytochemistry is described in Chapter 3.2.

Results of NGC I, NGC II and NGC III staining in cells of the different histological preparations are summarized in Table 5-2. Staining patterns do not show existence of blood group antigens for the same immunohistological results are obtained with stomachs of blood group donors A, B, AB and O (H).

In normal gastric mucosa of the corpus and the antrum, NGC I is confined to the cytoplasm of surface epithelial cells and the mucus covering its surface; little NGC I is found in the neck area (Fig. 5-2). In cases of single goblet cell metaplasia, this staining pattern is maintained although mucus production of surface epithelia is altered. This is characterized by the formation of epithelial cells with goblet cell-like appearance and positive reaction for NGC I. However, these goblet cell-like cells functionally are not goblet cells because they lack a typical intestinal goblet cell glycoprotein, namely NGC III (reference to this is made below). Cells of the original surface epithelium often become heavily compressed so that they progres-

* Collaborative study with Dr. Wurster from the Institute of Pathology of the City-Hospital, München-Schwabing.

Table 5-2: Immunohistological Pattern of Antigenic Neutral Glycoconjugates (NGC) in Normal Human Stomach and Defined Diseases.

Localization	NGC I	NGC II	NGC III
Surface epithelial cells	+[a]	Ø	Ø
Isthmus mucus cells	(+)/Ø	(+)	Ø
Mucus neck cells	Ø	+	Ø
Parietal cells	Ø	Ø	Ø
Chief zymogenic cells	Ø	Ø	Ø
Antral gland cells	Ø	+	(+)
Single goblet cell metaplasia			
"goblet" cells	+	Ø	Ø
dyspl. surface epith. cells	(+)/Ø	Ø	Ø
Intestinal metaplasia			
(differentiated type)			
goblet cells	Ø	(+)	+
columnar cells	(+)/Ø	Ø	Ø
Intestinal metaplasia			
(proliferating type)			
goblet cells	Ø	(+)	+
columnar cells	(+)/Ø	Ø	Ø

[a] Staining reaction: Ø no; (+) faint; + strong

sively loose the morphological characteristics of surface epithelial cells. Mucus producing columnar cells disappear. Merely, dysplastic surface epithelium with apparent functional and structural atrophy is observed. At this stage, NGC I staining diminuishes or even disappears while histological areas of intact surface epithelium strongly stain for NGC I.

In intestinal metaplasias of both differentiated and proliferating types, goblet cells do not react for NGC I. Occasionally, very faint reactions occur in striated columnar cells (Fig. 5-3). In contrast to single goblet cell metaplasia, goblet cells and striated columnar cells in intestinal metaplasia are not directly derived from surface epithelium. Histogenetically, intestinal metaplasia of the gastric mucosa is thought to arise from cells of the neck area [39, 47, 48). In intestinal metaplasia undifferentiated cells will develop into both goblet cells and striated columnar cells. In consideration of this phenomenon, the observed staining of NGC I becomes understandable. Immunohistology provides sensitive parameters in the assessment of loss and gain of distinct cellular molecules and will enable more precise investigations of cell differentiation.

Trace amounts of NGC II are observed in isthmus mucus cells and large amounts occur in mucus neck cells of normal gastric mucosa of the corpus and the antrum; antral gland cells also stain (Fig. 5-4, 5-5). No staining for NGC II is observed in either normal surface epithelial cells, cells of single goblet cell metaplasia or cells having a dysplastic appearance with functional atrophy.

In goblet cells of intestinal metaplasia (differentiated and proliferating types), this neutral glycoprotein becomes (if ever) only faintly expressed. When intestinal metaplasias are detected in histological sections, faint NGC II staining can coincide with the emergence of goblet cells. Then, goblet cells are seen as single cells, focal collections or in diffuse patterns.

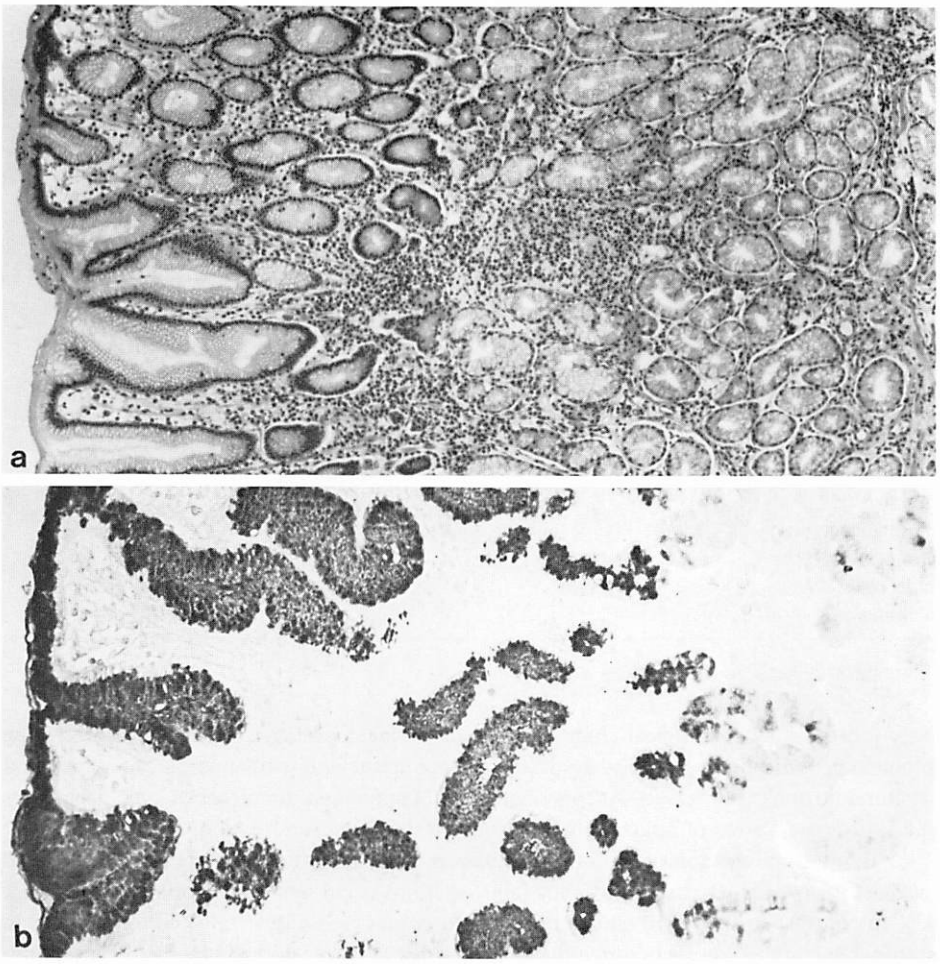


Figure 5-2. Human stomach, antrum. (a) Hematoxylin-eosin stain, original × 63. (b) Antral mucosa with slight inflammatory changes. Positive NGC I reaction of surface epithelial cells and of mucus covering the surface. NGC I staining is fading in the neck area of pyloric glands, original × 160.

Mitotic activity in normal gastric mucosa is mainly confined to cells of the neck cell area, and neck cells play a major role in replacing epithelial cells. Moreover, histogenetic relationships exist between neck cells, chief zymogenic cells and parietal cells [49]. The NGC II positive cells may, thus, be considered as precursor cells which in the course of physiological migration/differentiation towards surface epithelium loose NGC II and gain NGC I. During migration/differentiation into the opposite direction cells loose NGC II and express new molecular specificities e.g. zymogen, which is detectable by immuno-staining of pepsinogen (Table 5-1). In contrast, differentiated mucus producing pyloric gland cells will maintain NGC II synthesis.

NGC III is a typical product of goblet cells in normal intestinal mucosa (Fig. 5-6). In normal gastric mucosa, only faint NGC III staining of antral gland cells is found; other cells

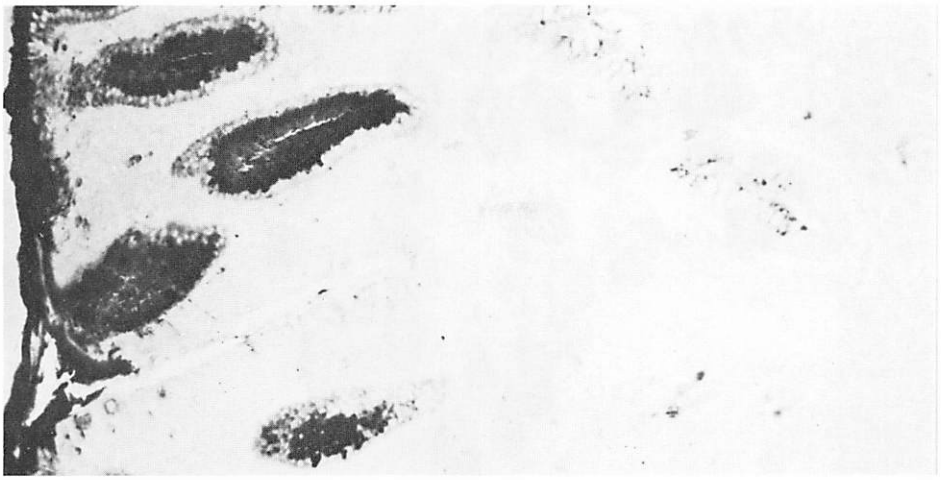


Figure 5-3. Antral mucosa with partial gland atrophy in chronic inflammation. NGC I reactions are detected in surface epithelium cells but not in goblet cells of intestinal metaplasia. Note some faint NGC I reactions in columnar striated cells, original × 160.

do not stain. Large amounts of NGC III are present in goblet cells of intestinal metaplasia i. e. in both differentiated and proliferating types. Single goblet cells or foci of these are clearly stained within gastric mucosa, which often shows chronic gastritis and partial gland atrophy; mucus in crypt's lumina also stains (Fig. 5-7).

Most interestingly, in single goblet cell metaplasia of surface epithelium NGC III is not detected while NGC I is still present in large quantities. As stated above, NGC I is not detected in goblet cells of intestinal metaplasias. This observation is interpreted in that goblet

Figure 5-4. Normal human stomach, corpus. NGC II staining occurs in isthmus mucus cells and in mucus neck cells of chief glands; weak reactions with luminal content of glands, original × 160. Inset: higher magnification view of NGC II staining in mucus neck cells (←), original × 540.

Figure 5-5. Antral mucosa; localization of NGC II in pyloric gland cells, original × 160.

cells of single goblet cell metaplasia do not possess the molecular components and functional state of intestinal goblet cells while maintaining at least some characteristics of normal gastric surface epithelium. Thus, there is a qualitative difference between goblet cells from single goblet cell metaplasia and those of intestinal metaplasia.

Detailed immunohistological studies and their evaluation in consideration of data from other authors which employ immunohistology and classical histochemistry are still in progress. We are aware that more detailed studies are needed for a concise description of physiological and pathological cell differentiation and especially for the elucidation of cancer development and its diagnosis in early stages. To date an ideal marker could not be found. Hitherto known and described marker systems suffer from the drawback that they become

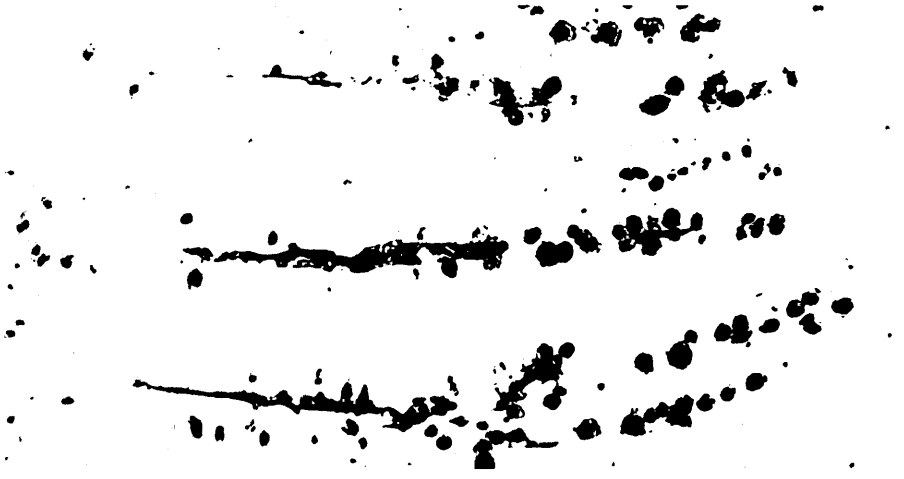


Figure 5-6. Human duodenal mucosa. Localization of NGC III in goblet cells and between villi, original × 160.

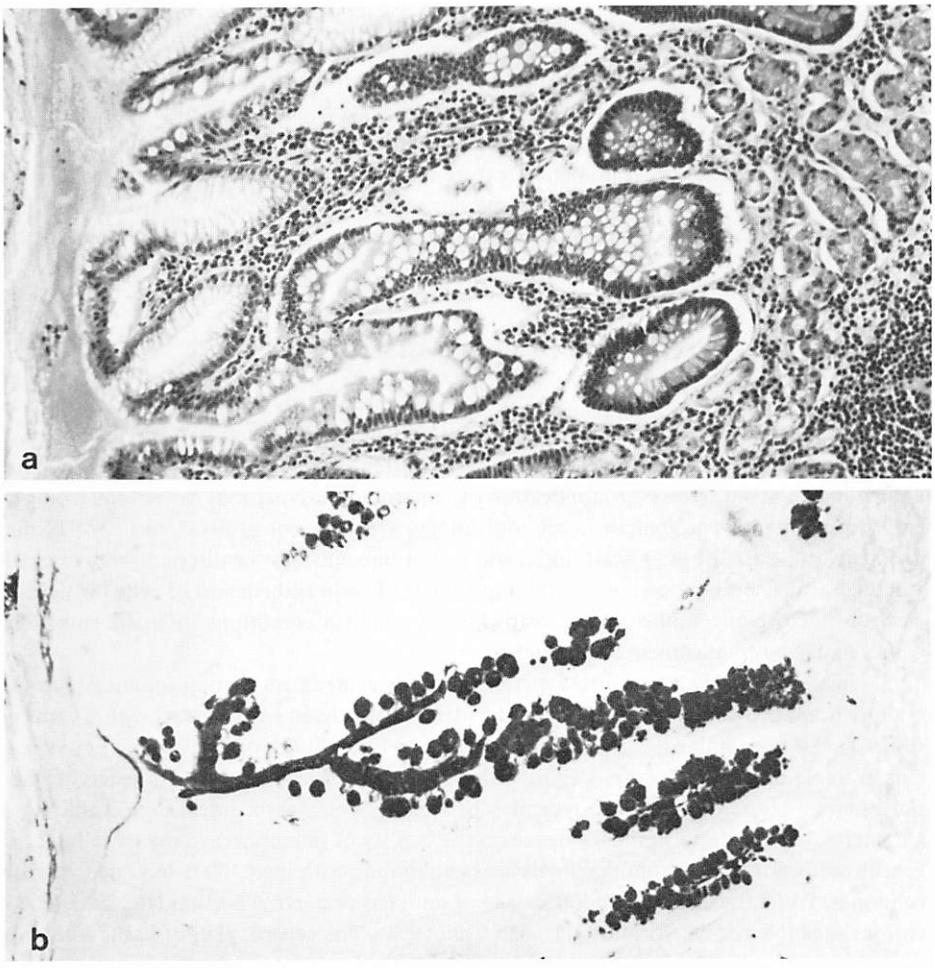


Figure 5-7. Foci of intestinal metaplasia in chronic gastritis with partial gland atrophy of antral mucosa. (a) Hematoxylin-eosin stain, original × 160. (b) NGC III staining of goblet cells and the crypt's mucus content, original × 160.

detectable only in relatively late stages. Moreover, and very importantly, malignancy is often associated with the development of highly heterogeneous cell populations and heterogeneous expression of molecular specificities.

An important step in this direction would be further characterization of isolated NGC molecules, e.g. amino acid and carbohydrate analyses, immunological typing (by monoclonal antibodies, immunological absorption studies), detailed blood group typing. Sugar specific lectins might appear to be useful tools as well. Analogous to antibody-enzyme labeling, lectins can also be conjugated with selected enzymes and employed in combination or in parallel with immunohistology. The use of cross-reacting antibodies (anti-human NGCs) for the description of homologous substances in the animal gastrointestinal tract would be another interesting project. The selection of an appropriate animal model for experiments on the develop-

ment of defined mucosa lesions (including cancer) could illustrate the developments in the neck cell area during carcinogenesis.

It can be expected that retrospective immunohistological studies with formerly prepared and embedded material and follow-up studies with endoscopically derived material (biopsies) will be valuable for prognosis and useful for defining premalignant stages. In this context, the application of histological texture analysis (i. e. quantitative image analysis of cells for determination of cell-associated parameters) will provide new data. Cell-associated molecules released into body fluids could be quantitated and correlated with defined disease.

5.3 *Antigen Detection in Semithin Resin Sections*

Though the paraffin technique proves useful for immuno-staining, specimen preparation according to electron microscopic criteria is often preferable because structural details are better preserved. However, application of immunological methods to sections from resin embedded organs is a goal in immunohistology which is not always reached. Technical problems still exist for large scale applications, but one must not be discouraged by negative staining results. Because of the general importance of resin embedment of cells for light and electron microscopic studies, it is worthwhile to establish conditions of tissue embedment which enable postembedment immunohistology.

In a recent study we discussed methods for postembedment immunostaining [50]. Generally, optimal fixation is first determined by trial. Then, resin embedments with a variety of resins should be examined, e.g., with the widely used Epon 812 or other available epoxy resins (for example diepoxide octane, DEO), with the polar methacrylate based Lowicryl K4M or polyester (Vestopal) resins; for typical schedules of resin embedments see Table 4-3 in Chapter 4.3. Furthermore, the influence on the quality of immunoreactivity of cellular antigens in sections may be examined by tissue conditioning with inert fillers (e.g. polyvinylpyrrolidones, PVP), by chemical modification of antigens (e.g. ethyl acetimidate, EAI) and by various schedules of dehydration (cf. also Chapter 4). The general plan of tissue treatments which we have tested is summarized in Table 5-3.

Table 5-3: Tissue Treatments for Postembedment Immunohistology with Resin Sections.

Fixation	Protective agents	Dehydration[a)]	Embedment[b)]	Immunohistology
Formaldehyde	–	ethanol or eth. glycol	Epon 812	+
			Epon DEO	(+)
			Lowicryl K4M	–/(+)
			Vestopal	(+)
Formaldehyde	5 – 10 % PVP	ethanol	Epon 812	+ +
Formaldehyde	0.2 mol/L ethyl acetimidate	ethanol	Epon 812	+ +

[a)] Schedules of dehydration see Table 4 – 2.
[b)] Schedules of resin embedments see Table 4 – 3.

It was found that a broader spectrum of antigens than expected can be localized by postembedment immunohistology of resin embedded organs. In our laboratory, a variety of molecules synthesized by liver, gastrointestinal mucosa and lymphoid tissue are currently stained in sections from the respective tissues embedded in Epon 812 (Table 5-4). Apart from such target antigens, other biomolecules of histopathological relevance can be stained, too (Chapter 6.2).

In the following, we give a typical protocol for the immunolocalization of antigens in semithin Epon sections. The detection of AFP in regenerating mouse liver is taken as model.

Table 5-4: Immunoperoxidase Localization of Antigens in Sections from Epon Embedded Organs.

Tissue	Target antigen	Substance class	Localization
Liver	albumin	serum protein	hepatocytes
	α_1-fetoprotein	oncofetal antigen	hepatocytes, carcinoma
	epoxide hydrolase	microsomal enzyme	hepatocytes
Stomach	IgA, IgG	immunoglobulins	plasma cells
	pepsinogen I	proenzyme	chief cells neck cells
	neck cell antigen	mucus glycoconjugate, PAS stainable	neck cells
	goblet cell antigen (GOA/AB)	mucus glycoconjugate, Alcian blue stainable	goblet cells
	goblet cell antigen (GOA/PAS)	mucus glycoconjugate, PAS stainable	goblet cells
	carcinoembryonic antigen (CEA)	oncofetal antigen	striated cells, carcinoma
	gastrin	peptide hormone	G-cells
Colon	GOA/AB GOA/PAS	mucus glycoconjugates, Alcian blue or PAS stainable	goblet cells
	CEA	oncofetal antigen	striated cells, carcinoma
Lymph node	IgG, IgM	immunoglobulins	plasma cells

Protocol of immuno-staining:

1. Tissue sampling: livers are sliced into cubes of about 2 mm and fixed at 0°C in 6 % formaldehyde made in 0.2 mol/L cacodylate buffer pH 7.2 for 6 – 8 h and washed overnight at 0°C with several changes of the buffer solution. Prior to dehydration by increasing series of either ethanol at 0°C or ethylene glycol, a batch of tissue blocks is first conditioned with 5 – 10 % PVP in buffer for 2 h. Dehydration is then performed in ethanol supplemented with PVP. Another batch of tissue blocks is treated with 0.2 mol/L ethyl acetimidate – HCl in 0.2 mol/L K_2HPO_4 (pH adjusted to pH 7.3) for 2 h at 0 – 4°C [50] before dehydration is started. Dehydration and resin embedments were done according to Tables 4-2 and 4-3. 1 – 1.5 μm thick sections are deposited on acetone cleaned slides and dried at 90°C for 20 minutes.

2. Pretreatment: resin is partially removed with sodium methoxide according to Mayor et al. [51]. Then, preparations are passed into 10 % H_2O_2/PBS for 10 min in order to inhibit endogenous peroxidases. Sections are rinsed again in PBS (3 x 2 min) followed by BSA/PBS for 5 minutes. All sera are made up in BSA/PBS.
3. Incubation: in principle as described above (Chapter 5.1) for method I and method II.
4. Enzymo-cytochemical detection of HRP: DAB cytochemistry according to Chapters 3.3 and 5.1. Finally, sections are dehydrated and mounted under coverglass.

AFP is readily stained in sections when aldehyde fixed liver blocks are submitted to resin embedment. Certain treatments after aldehyde fixation and certain resin embedments improve the immunohistological reactions. Thus, conditioning of tissue blocks with PVP or amidination of amino groups by EAI prior to resin embedment give reliable and strong staining (Fig. 5-8, Table 5-3). We are aware that other antigens will behave quite differently and especially the protective effect (PVP, EAI) will probably vary from one antigen to another. Then, alternative procedures must be elaborated.

One reason for successful immuno-staining of antigens in resin sections is the adoption of appropriate tissue sampling, i.e. fixation, dehydration, embedment. For general rules of the latter see Chapter 4. Furthermore, incubation schedules must be carefully established with optimally prepared immunohistological reagents. Another reason is the partial removal of resin from the section. This step must be well executed so that antigenic determinants become sufficiently exposed.

When epoxy monomers are cured, three-dimensional structures are formed and cross-links are principally based on di-ester and ether bridges [52, 53]. Hence, these must be partially broken up and dissolved in order to give access to immunological reagents for reaching antigenic sites. Various procedures exist for this approach and their effect on immuno-staining of antigens must be examined in the respective histological preparations (Table 5-5). It was found that alcoholic sodium hydroxide as well as sodium methoxide in methanol/benzene enable satisfactory immunolocalization of antigens like AFP. In the meantime we have studied numerous other antigens in semithin resin sections for which these "etching" procedures prove also useful.

Sodium methoxide in a mixture of methanol and benzene was the first described method [51] and it is still one of the most powerful reagents which break up at least di-ester bridges. We suppose that after such a reaction subsequent washings of sections in methanol/benzene and acetone will also remove unreacted monomers and oligomers. The latter may occur in cured resins, moreover, not fully reacted compounds are present in castings when Epon is cured in steps of increasing temperatures [56]. In view of this, we prefer the curing of our tissues at 35°C/24 h, then at 45°C/24 h and finally at 60°C/24 even if direct curing at 60°C/24 h also yields sections in which immuno-staining is well performed. The heat liberated in the course of polymerization seems not to impair antigenicity.

We have no evidence that H_2O_2 alone will break up and remove sufficiently polymerized Epon from semithin sections. The same holds true for treatments simply with absolute ethanol, methanol or acetone. Yet, we must keep in mind that it is not necessary in all cases to remove (or partially remove) polymerized resin for successful immuno-staining. For example, peptide hormones can be well located when sections are only treated with H_2O_2 [57].

AFP stains better in sodium methoxide treated Epon 812 than in similarly treated DEO or Vestopal sections (see Table 5-3). The reason for that is not yet understood. Neither in

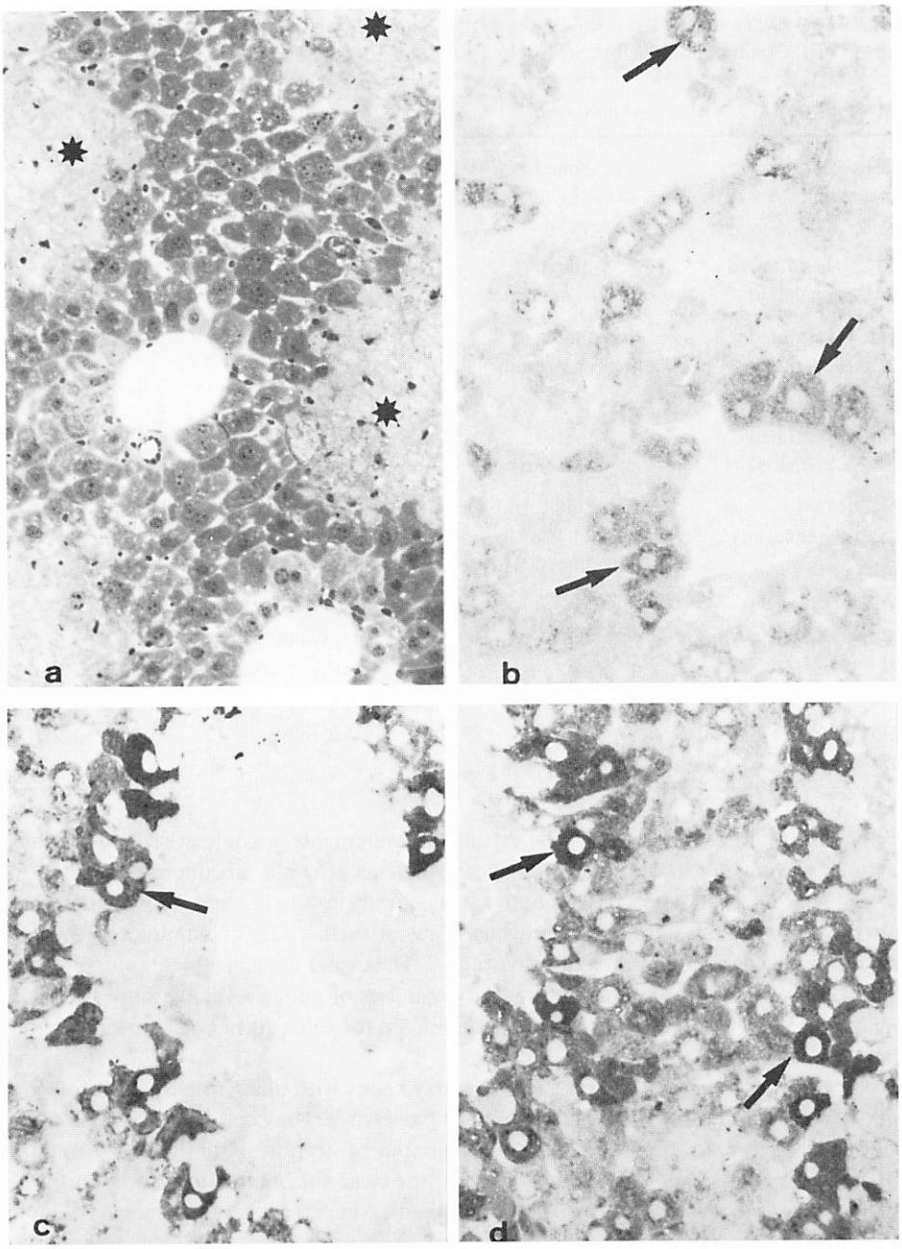


Figure 5-8. Postembedment immunolocalization of AFP in semithin section; formaldehyde fixation and Epon embedment of regenerating mouse liver after CCl_4 intoxication. AFP is seen in non-damaged hepatocytes (←). (a) Azur II stain, necrotic area (*), original × 160. (b) Section from same tissue block stained for AFP, original × 250. (c) Aldehyde fixed liver block was conditioned with 10 % PVP prior to dehydration and resin embedment, original × 250. (d) Aldehyde fixed liver block was treated with EAI prior to dehydration and resin embedment, original × 250 (from W.D. Kuhlmann and R. Krischan, *Histochemistry* **72** [1981] 377).

Table 5-5: Examples for Removal of Resin from Semithin Sections of Epon Embedded Liver and the Effect on the Immunolocalization of AFP by Use of Peroxidase Labeled Antibodies.

Method	Application	Exposure	Immunohistology
CH_3ONa (5 %) in methanol/benzene [51]	1:2 diluted in methanol/benzene	2–4 min	++
CH_3ONa saturated in abs. methanol	1:2 diluted in abs. methanol	2–4 min	(+)/+
NaOH saturated in abs. ethanol [54]	1:2 diluted in abs. ethanol	5–10 min	++
NaOH saturated in abs. methanol	1:2 diluted in abs. methanol	5–10 min	++
NaOH (2 %) in abs. ethanol	not diluted	5 min	++
	1:2 diluted in dist. water	5 min 20 min	∅/(+) +
abs. ethanol abs. methanol abs. acetone	not diluted	10–20 min	∅
H_2O_2 [55]	5–10 % in PBS or H_2O	5–20 min	∅

Epon nor in other embedments were distinct measurements possible in order to calculate the amount of antigenic determinants which can still react after all embedding steps. In the course of resin curing, monomers and oligomers will not only lead to linkages between them but may also react with amino groups of antigens. Thus, a further loss of immunoreactivity will be added to that due to fixation and dehydration. The extent of such a loss is still unknown. At all events, we must expect that substantial quantities of antigen reactive sites/regions are no longer reactive. Yet, sufficient numbers can remain for subsequent postembedment immunohistology.

In order to reduce the reaction of epoxy resins with antigens we can introduce acetimidyl groups by EAI treatment into tissue antigens or we may employ PVP as an unreactive filler (Chapter 4.4). Both measures result in the protection of antigens to a certain yet undefined extent. Such treatments are useful for clear-cut immunolocalization of AFP and other antigens in a variety of organs (unpublished). Apart from partial removal of Epon from sections, sodium methoxide will also displace acetimidyl groups from the antigens in EAI-reacted livers. Interestingly, further removal of acetimidyl groups by ammonia-acetic acid [58] and regeneration of the original amino groups seem not to be important because equally strong immunohistological reactions are seen in reversed and non-reversed sections.

In contrast to epoxy embedment, only very faint immuno-staining is observed in sections from K4M embedded liver. Dehydration per se at 0°C was certainly not the reason for that pitfall because dehydration of tissue for Epon embedment followed the same schedule.

One objection may be that in our experiments K4M is cured at 0°C. Apart from a different type of cross-linking in K4M than in Epon 812 embedment, the influence of curing temperature on tissue antigens must be reconciled. Lowering the temperature will probably reduce denaturation in K4M embedment. In view of publications with Protein A – gold complex as marker where K4M is reported to be a suitable embedding medium for postembedment immunohistology [59], low-temperature dehydration and embedment in polar resin hold great promise for future postembedment immunohistology. Many of the above objections hold true for postembedment immuno-electron microscopy.

5.4 *Immunolocalization of Antigens in Monolayer Cultures*

Cultivation of cells *in vitro* by the monolayer technique is an important tool in all areas of biomedicine, and immunohistological analysis enables in situ detection and characterization of cell-associated molecules without mechanical disturbance of the cultured cells. Monolayer cell cultures may be grown either in plastic petri dishes or on glass cover slips, and, in contrast to solid tissues, such cultures do not need previous dehydration and embedment for immuno-staining of intracellular antigens. These can be immediately stained after appropriate cell stabilization. We describe here the staining protocol for the localization of T-antigen in SV-40 transformed cells. It is not intended to give a concise description of its intracellular distribution. Merely, preparative steps are shown which were studied in collaboration with Dr. H. G. Suarez and A. Viron from the Institut de Recherches Scientifiques sur le Cancer in Villejuif/France.

Protocol of immuno-staining:

1. Tissue sampling: prior to treatment with antibodies, cultures grown on glass cover slips are rinsed in PBS and transferred into fixatives. Results from a comparative study are summarized in Table 5-6. For light microscopical studies, fixation in acetone proves best [60]. To this aim, cultures are placed into ice-cold absolute acetone for 5 min and are warmed up to room temperature within 2 min followed by air-drying. Fixed and air-dried cultures are immediately used for immuno-staining; alternatively, they are stored at – 70°C until use.
2. Pretreatment: cultures are washed in PBS for 5 min followed by BSA/PBS.
3. Incubation: the indirect peroxidase labeled antibody method is described here. Of course, other incubation procedures are also possible (see Chapter 3.1). Cultures are covered with the respective sera and incubated at room temperature in a humid chamber;
- rabbit normal serum (1:30 diluted in BSA/PBS) for 5 min followed by rinsing in BSA/PBS;
- first step, hamster anti-T (whole immune serum, γ-globulin fraction or isolated antibodies), concentrations are determined by trial as described in Chapter 5.1 for method I; incubation time is usually 20-30 min followed by washings in BSA/PBS (3 x 5 min);
- second step, rabbit anti-hamster IgG labeled with HRP (0.01-0.1 mg/ml) for 20 min followed by washings in PBS (3 x 5 min).

4. Enzymo-cytochemical detection of HRP: see Chapter 3.2 and 5.1; cultures are dehydrated and mounted upside down on microscope slides.

Typical examples for T-antigen localization are shown in Fig. 5-9. The neoantigen is located in the nucleus and appears to be finely granulate. The nucleolus is devoid of T-antigen. Apart from the usual fixation in cold absolute acetone, fixation in low concentrations of aldehydes

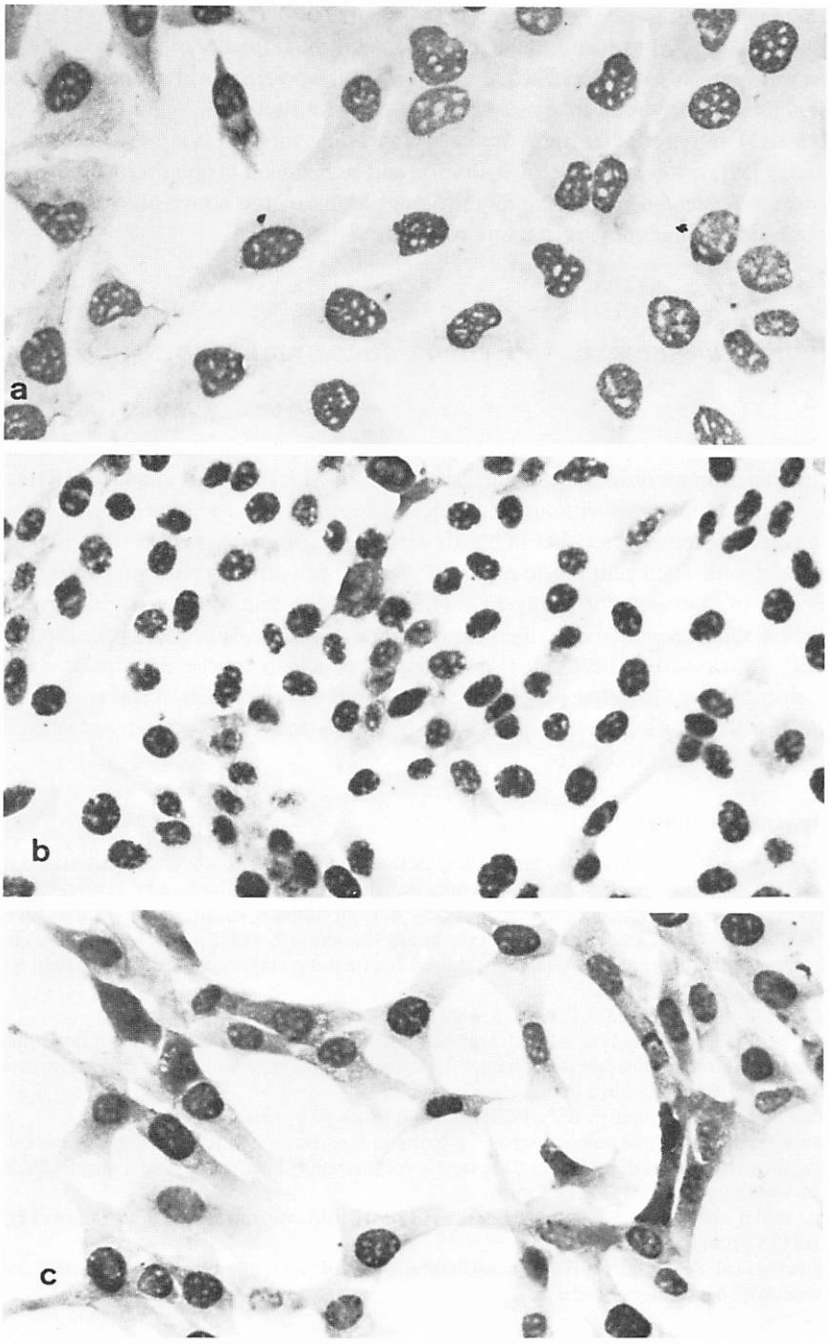


Figure 5-9. Localization of T-antigen in SV-40 transformed cells (monolayer cultures); (a) acetone fixed WI 98 VdD human skin cells; (b) acetone fixed SV 3T3 mouse fibroblasts; (c) 4 % formaldehyde/30 min fixed SV 3T3 mouse fibroblasts. Original × 250.

for short periods also give satisfactory immunohistological results. Yet, cells tend to detach easily. Higher concentrations of aldehydes and longer fixation periods which are often desirable for ultrastructural studies lead to considerable loss of immunoreactivity (Table 5-6). In the meantime, the expression of T-antigen in transformed cells has been well demonstrated by fluorescent and peroxidase techniques.

Table 5-6: Influence of Tissue Fixation on T-Antigen Detection.

Tissue fixation	Light microscopy		Electron microscopy
Monolayer cultures	ICC staining[a]	% nuclei positive[b]	Cell conservation
Acetone 5 min	+	> 90	not done
1 % Formaldehyde 30 min	+	–	not done cells detached
4 % Formaldehyde 5 min	+	–	not done cells detached
4 % Formaldehyde 30 min	+	> 90	sufficient
0.2 % Glutaraldehyde 1 min	(+)/+	70	sufficient
0.2 % Glutaraldehyde 15 min	(+)[c]	20	good
0.5 % Glutaraldehyde 1 min	(+)[c]	20	good
2 % Glutaraldehyde 15 min	∅	∅	excellent
4 % Form. – 0.2 % Glut. 15 min	(+)[c]	20	good

[a] Intensity of specific reactions: ∅ none; (+) faint; + strong
[b] Mean from 3 experiments ± 10 %; – not counted, cells detached
[c] Faint reactions and many negative islands

References

1. C. R. Taylor and G. Kledzik, "Immunohistologic techniques in surgical pathology. A spectrum of "new" special stains", *Human Pathol.* **12** (1981) 590.
2. B. Romeis: *Mikroskopische Technik*. R. Oldenbourg, München-Wien 1968.
3. W. D. Kuhlmann, "Purification of mouse alpha$_1$-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization", *Histochemistry* **44** (1975) 155.
4. R. C. Nairn, J. E. Fothergill, M. G. McEntegart and H. G. Richmond, "Loss of gastro-intestinal-specific antigen in neoplasia", *Br. Med. J.* **2** (1962) 1791.
5. R. C. Nairn and W. G. R. M. De Boer, "Species distribution of gastrointestinal antigens", *Nature* **210** (1966) 960.

6. W. G. R. M. De Boer, A. Forsyth and R. C. Nairn, "Gastric antigens in health and disease. Behaviour in early development, senescence, metaplasia, and cancer", *Br. Med. J.* **3** (1969) 93.
7. P. Gold and S. O. Freedman, "Specific carcinoembryonic antigens of the human digestive system", *J. Exp. Med.* **122** (1965) 467.
8. S. von Kleist and P. Burtin, "Localisation cellulaire d'un antigène embryonnaire de tumeurs coliques humaines", *Int. J. Cancer* **4** (1969) 874.
9. I. Häkkinen, O. Järvi and J. Grönroos, "Sulphoglycoprotein antigens in the human alimentary canal and gastric cancer. An immunohistological study", *Int. J. Cancer* **3** (1968) 572.
10. H. Kawasaki, K. Imasato, E. Kimoto, K. Akiyama and M. Takeuchi, "Immunohistological studies on intestinal mucosa-specific glycoprotein in gastric carcinoma", *Gann* **63** (1972) 231.
11. H. Kawasaki and E. Kimoto, "Mucosal glycoproteins in carcinoma cells of gastrointestinal tract, as detected by immunofluorescence technique", *Acta Path. Jap.* **24** (1974) 481.
12. I. Jabbal, D. I. C. Kells, G. Forstner and J. Forstner, "Human intestinal goblet cell mucin", *Can. J. Biochem.* **54** (1976) 707.
13. D. V. Gold and F. Miller, "Characterization of human colonic mucoprotein antigen", *Immunochemistry* **11** (1974) 369.
14. D. V. Gold, Immunoperoxidase localization of colonic mucoprotein antigen in neoplastic tissues", *Cancer Res.* **41** (1981) 767.
15. D. M. Goldenberg, K. D. Pant and H. L. Dahlman, "Antigens associated with normal and malignant gastrointestinal tissues", *Cancer Res.* **36** (1976) 3455.
16. W. Rapp, M. Windisch, P. Peschke and K. Wurster, "Purification of human intestinal goblet cell antigen (GOA), its immunohistological demonstration in the intestine and in mucus producing gastrointestinal adenocarcinomas", *Virchows Arch. (Pathol. Anat.)* **382** (1979) 163.
17. J. Bara, F. Loisillier and P. Burtin, "Antigens of gastric and intestinal mucous cells in human colonic tumours", *Br. J. Cancer* **41** (1980) 209.
18. J. Ma, W. G. R. M. De Boer and J. Nayman, "Intestinal mucinous substances in gastric intestinal metaplasia and carcinoma studied by immunofluorescence", *Cancer* **49** (1982) 1664.
19. J. Forstner, N. Taichman, V. Kalnins and G. Forstner, "Intestinal goblet cell mucus: isolation and identification by immunofluorescence of a goblet cell glycoprotein", *J. Cell Sci.* **12** (1973) 585.
20. R. Spee-Brand, G. J. A. M. Strous and M. F. Kramer, "Isolation and partial characterization of rat gastric mucous glycoprotein", *Biochim. Biophys. Acta* **621** (1980) 104.
21. H. L. Smits, P. J. M. van Kerkhof and M. F. Kramer, "Isolation and partial characterization of rat duodenal-gland (Brunner's-gland) mucus glycoprotein", *Biochem. J.* **203** (1982) 779.
22. A. E. Szulman, "The histological distribution of blood group substances A and B in man", *J. Exp. Med.* **111** (1960) 785.
23. A. E. Szulman, "The histological distribution of the blood group substances in man as disclosed by immunofluorescence. II. The H antigen and its relation to A and B antigens", *J. Exp. Med.* **115** (1962) 977.
24. G. W. Slocombe, C. L. Berry and K. V. Swettenham, "The variability of blood group antigens in gastric carcinoma as demonstrated by the immunoperoxidase technique", *Virchows Arch. (Pathol. Anat.)* **387** (1980) 289.
25. A. Kapadia, T. Feizi, D. Jewell, J. Keeling and G. Slavin, "Immunocytochemical studies of blood group A, H, I, and i antigens in gastric mucosae of infants with normal gastric histology and of patients with gastric carcinoma and chronic benign peptic ulceration", *J. Clin. Pathol.* **34** (1981) 320.
26. I. M. Samloff, "Cellular localization of group I pepsinogens in human gastric mucosa by immunofluorescence", *Gastroenterology* **61** (1971) 185.
27. I. M. Samloff and W. M. Liebman, "Cellular localization of the group II pepsinogens in human stomach and duodenum by immunofluorescence", *Gastroenterology* **65** (1973) 36.
28. W. D. Kuhlmann, H. Fritsch and W. Rapp, "Immunfluorescenzhistologischer Nachweis der antigenen Magenschleimhautesterase VI A in dem Oberflächenepithel der menschlichen Korpusschleimhaut", *Z. Ges. Exp. Med.* **152** (1970) 93.
29. W. Rapp and K. Wurster, "Gastric marker proteins. Purification and immunohistological demonstration of the chief cell esterase", *Virchows Arch. (Pathol. Anat.)* **390** (1981) 151.
30. H. Denk, G. Tappeiner, A. Davidovits and J. H. Holzner, "The carcinoembryonic antigen (CEA) in carcinomata of the stomach", *Virchows Arch. (Pathol. Anat.)* **360** (1973) 339.

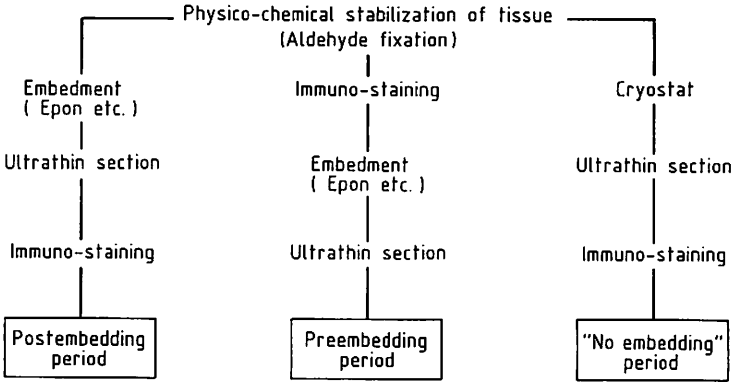
31. D. M. Goldenberg, R. M. Sharkey and F. J. Primus, "Carcinoembryonic antigen in histopathology: immunoperoxidase staining of conventional tissue sections", *J. Natl. Cancer Inst.* **57** (1976) 11.
32. W. Rapp and K. Wurster: "The CEA positive cancer and non cancer cells of the stomach. Diagnostic and histogenetic significance". In: F. G. Lehmann (ed.): *Carcino-Embryonic Proteins,* vol. 2. Elsevier-North-Holland and Biomedical Press, Amsterdam-London-New York 1979.
33. G. Bussolati and A. G. E. Pearse, "Immunofluorescent localization of the gastrin-secreting G cells in the pyloric antrum of the pig", *Histochemie* **21** (1970) 1.
34. J. M. Polak, B. Stagg and A. G. E. Pearse, "Two types of Zollinger-Ellison syndrome: immunofluorescent, cytochemical and ultrastructural studies of the antral and pancreatic gastrin cells in different clinical states", *Gut* **13** (1972) 501.
35. P. Brandtzaeg, "Mucosal and glandular distribution of immunoglobulin components. Immunohistochemistry with a cold ethanol-fixation technique", *Immunology* **26** (1974) 1101.
36. R. W. Mowry, "The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with the periodic acid-Schiff reaction", *Ann. N. Y. Acad. Sci.* **106** (1963) 402.
37. R. Lev and S. S. Spicer, "Specific staining of sulphate groups with alcian blue at low pH", *J. Histochem. Cytochem.* **12** (1964) 309.
38. S. S. Spicer, "Diamine methods for differentiating mucosubstances histochemically", *J. Histochem. Cytochem.* **13** (1965) 211.
39. M. I. Filipe, "Mucins in the human gastrointestinal epithelium: a review", *Invest. Cell Pathol.* **2** (1979) 195.
40. W. D. Kuhlmann and W. Rapp, "Über die Artspezifität der Magenschleimhautesterase VI A des Menschen. I. Vergleichende Untersuchungen über Magenschleimhautextrakte von Kaninchen, Meerschweinchen, Ratte und Mensch", *Z. Immunitätsforsch.* **140** (1970) 9.
41. G. E. Konjetzny: "Die Entzündungen des Magens". In: F. Henke and O. Lubarsch (eds): *Handbuch der speziellen pathologischen Anatomie und Histologie,* vol. IV/2. Springer-Verlag, Berlin-Heidelberg-New York 1928.
42. R. Lev, "The mucin histochemistry of normal and neoplastic gastric mucosa", *Lab. Invest.* **14** (1966) 2080.
43. H. Goldman and S. C. Ming, "Fine structure of intestinal metaplasia and adenocarcinoma of the human stomach", *Lab. Invest.* **18** (1968) 203.
44. J. R. Jass and M. I. Filipe, "A variant of intestinal metaplasia associated with gastric carcinoma: a histochemical study", *Histopathol.* **3** (1979) 191.
45. J. R. Jass, "Role of intestinal metaplasia in the histogenesis of gastric carcinoma", *J. Clin. Pathol.* **33** (1980) 801.
46. P. Laurén, "The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification", *Acta Pathol. Microbiol. Scand.* **64** (1965) 31.
47. S. C. Ming, H. Goldman and D. G. Freiman, "Intestinal metaplasia and histogenesis of carcinoma in human stomach. Light and electron microscopic study", *Cancer* **20** (1967) 1418.
48. F. Iida, F. Murata and T. Nagata, "Histochemical studies of mucosubstances in metaplastic epithelium of the stomach, with special reference to the development of intestinal metaplasia", *Histochemistry* **56** (1978) 229.
49. W. Rubin, L. L. Ross, M. H. Sleisenger and G. H. Jeffries, "The normal human gastric epithelia. A fine structural study", *Lab. Invest.* **19** (1968) 598.
50. W. D. Kuhlmann and R. Krischan, "Resin embedment of organs and postembedment localization of antigens by immunoperoxidase methods", *Histochemistry* **72** (1981) 377.
51. H. D. Mayor, J. C. Hampton and B. Rosario, "A simple method for removing the resin from epoxy-embedded tissue", *J. Biophys. Biochem. Cytol.* **9** (1961) 909.
52. W. Fisch, W. Hofmann and J. Koskikallio, "The curing mechanism of epoxy resins", *J. Appl. Chem.* **6** (1956) 429.
53. H. Lee and K. Neville: *Handbook of Epoxy Resins.* McGraw-Hill, New York 1967.
54. B. P. Lane and D. L. Europa, "Differential staining of ultrathin sections of Epon-embedded tissues for light microscopy", *J. Histochem. Cytochem.* **13** (1965) 579.

55. P. K. Nakane, "Application of peroxidase-labelled antibodies to the intracellular localization of hormones", *Acta Endocrinologica (Kopenhagen) suppl.* **153** (1971) 190.
56. J. H. Luft, "Improvements in epoxy resin embedding methods", *J. Biophys. Biochem. Cytol.***9** (1961) 409.
57. G. C. Moriarty, "Adenohypophysis: ultrastructural cytochemistry, a review", *J. Histochem. Cytochem.* **21** (1973) 855.
58. M. L. Ludwig and R. Byrne, "Reversible blocking of protein amino groups by the acetimidyl group", *J. Am. Chem. Soc.* **84** (1962) 4160.
59. J. Roth, M. Bendayan, E. Carlemalm, W. Villiger and M. Garavito, "Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue", *J. Histochem. Cytochem.* **29** (1981) 663.
60. R. Wicker and S. Avrameas, "Localization of virus antigens by enzyme-labelled antibodies", *J. Gen. Virol.* **4** (1969) 465.

6 *Immunohistological Assays for Electron Microscopy*

While many approaches prove useful for light microscopy, numerous difficulties are encountered in ultrastructural immunohistology. The main goal of the latter, i.e. *in situ* characterization of cellular components and the relation between structure and function cannot usually be realized with a routine scheme. In order to understand false and correct staining results, different aspects must be considered. Apart from optimal preparation of immunohistological reagents (see Chapters 1 and 2), certain pitfalls in immuno-electron microscopy are especially linked with tissue fixation and processing. General points of specimen preparation can be read in Chapter 4 while we now consider practical aspects.

At the beginning of experimentation, we must evaluate the chosen model inasmuch as the cellular sites of antigens (e.g., in extracellular/intracellular spaces, cell surface membranes) are limiting factors in the choice of the marker substance and in the choice of the type of incubation (direct/indirect immuno-stainings, control reactions). Moreover, tissue sampling must be carefully examined. Here, tissue stabilization by adoption of a fixation method is usually the first step. For most purposes, tissue fixation is done by chemical processes, e.g. with aldehydes; details are described in Chapter 4.1. Then, ultrastructural immunolocalizations may be achieved by either of three different ways:



(a) Immuno-staining in postembedment period: fixed tissue blocks are dehydrated and embedded in one of the typical resins. Then, ultrathin sections are cut and immuno-stained.
(b) Immuno-staining in preembedding period: fixed tissue blocks are incubated in the different immunohistological reagents, postfixed (or not) in OsO_4, dehydrated and embedded in resin. Then, ultrathin sections are cut and examined in the electron microscope.
(c) Immuno-staining in "no-embedding" period: ultrathin frozen sections are cut from tissue blocks in a cryo-ultramicrotome and are submitted to immuno-staining. These preparations do not need conventional dehydration and resin embedment.

Immuno-staining may be difficult or even impossible to achieve on ultrathin sections of resin embedded tissues, and cryo-ultramicrotomy is not yet routine. Thus, many immunolocalizations must be performed by the so-called preembedment type. In any case, the optimal procedure needs to be established by trials, and specific cytological assays for a given biological model require individual developments. Important aspects of tissue sampling for ultrastructural immunohistology are given in the following section.

6.1 Immuno-staining in Preembedment Period

Subsequent to tissue fixation and prior to dehydration and resin embedment, cellular ligands are stained according to schedules shown in Chapter 3.1; intracellular antigens are preferably stained by *direct* procedures. It is well established that a compromise between ultrastructural preservation and retention of biological characteristics of the studied material must be made; for theoretical and practical aspects see Chapter 4.1. Yet, apart from loss of immunoreactivity due to fixation, negative staining results are also to be expected if immunohistological reagents are not able to reach their corresponding antigenic sites. Extensive studies which followed various fixation procedures made it clear that the localization of intracellular antigens by labeled antibodies is governed by their penetrability [1 – 3]. Freezing and thawing of slightly fixed (aldehyde) tissue blocks in the presence of labeled antibodies may enhance penetration ot the latter, but ultrastructural preservation of cells is very poor [1].

Cell surface membranes and intracellular membrane complexes are strong barriers in tissues and intact cells even if intracellular localization of antigens is achieved in single cell suspensions by use of Fab fragments conjugated with HRP [4]. Our studies with various markers of known molecular weight have shown that molecules of 80000 daltons and more do not penetrate cell membranes or do so only sluggishly. A series of experiments with various markers is summarized in Table 6-1.

In such experiments, rabbits were first immunized with one of the indicated markers; in the case of 7S antibody-HRP as marker, BSA immunized rabbits served as model. Due to the immunogenicity of the injected markers, specific antibody synthesis was initiated in lymphoid cells. Then, popliteal lymph nodes were excised from which single cell suspensions as well as small tissue fragments were prepared and fixed; cryostat sections were cut from the aldehyde fixed blocks. These tissue preparations were finally incubated with the corresponding markers and, due to their antigenic behavior, became bound by the corresponding intracellular antibodies. Of course, in the case of non-penetrability of the marker molecules, the

Table 6-1: Penetration of Macromolecules into Aldehyde Fixed Organs.

Marker molecules	Cell suspensions	Tissue blocks[a]	Cryostat sections
Peroxidase (HRP) mol wt 40000	+[b]	+	+
Alk. phosphatase mol wt 80000	Ø/(+)	Ø/(+)	+
Fragment Fab – HRP mol wt 80000	Ø/(+)	Ø/(+)	+
Glucose oxidase mol wt 190000	Ø	Ø/(+)	+
7S Antibody – HRP mol wt 200000	Ø	Ø/(+)	+

[a] Blocks are cut with razor blades, immunocytochemical stainings restricted to cells at the edge of the block

[b] Evidence for intracellular penetration of the markers, + staining in all expected compartments; (+) faint staining in few compartments; Ø no staining observed.

latter would not be bound. After appropriate washings and upon enzymo-cytochemical reaction, intracellular sites of specific antibodies (labeled by the marker) were finally revealed which permitted observations about the penetrability of markers with defined molecular weight.

Conclusions about the penetration of macromolecules into the cytoplasm and its endoplasmic reticulum are possible by such experiments. Thus, in aldehyde fixed lymph node cell suspensions, penetration of molecules of 80000 daltons reached its limit (Table 6-1 and Fig 6-1a). Enzymatic pre-digestion of cell surface material (cell-coat) with trypsin for a short time, with neuraminidase, papain or hyaluronidase and followed by aldehyde fixation (not shown in the Table) improved penetration of high molecular weight markers. However, the total number of stained cells always remained low. In all cases, the labeling success was limited most often only to few compartments of the rough-surfaced endoplasmic reticulum (RER). When examining the penetration into fixed tissue blocks, we observed that small hand-cut fragments were penetrated poorly if at all. As a rule, only cells at the edge of the block came into contact with immunohistological reagents. These cells, however, were not penetrated and cytological labelings were preferentially obtained in the extracellular space of superficial cell layers. Upon careful examination of such cells, positive immuno-staining could sometimes be seen in parts of the RER. These findings emphasize the penetration gradient for macromolecules due to membrane complexes. Reproducible localization of intracellular ligands could be regularly obtained when cryostat sections were employed (Table 6-1 and Fig. 6-1b).

In order to overcome such penetration problems and to make cells more permeable to conjugates and markers without significant impairment of morphology, treatments of aldehyde fixed cells with saponin, Tween and other detergents were proposed [5 – 9]. Indeed, this approach is useful for monolayer cell cultures or single cell suspensions inasmuch as the permeability of membranes is enhanced, but the deleterious effect of those treatments to

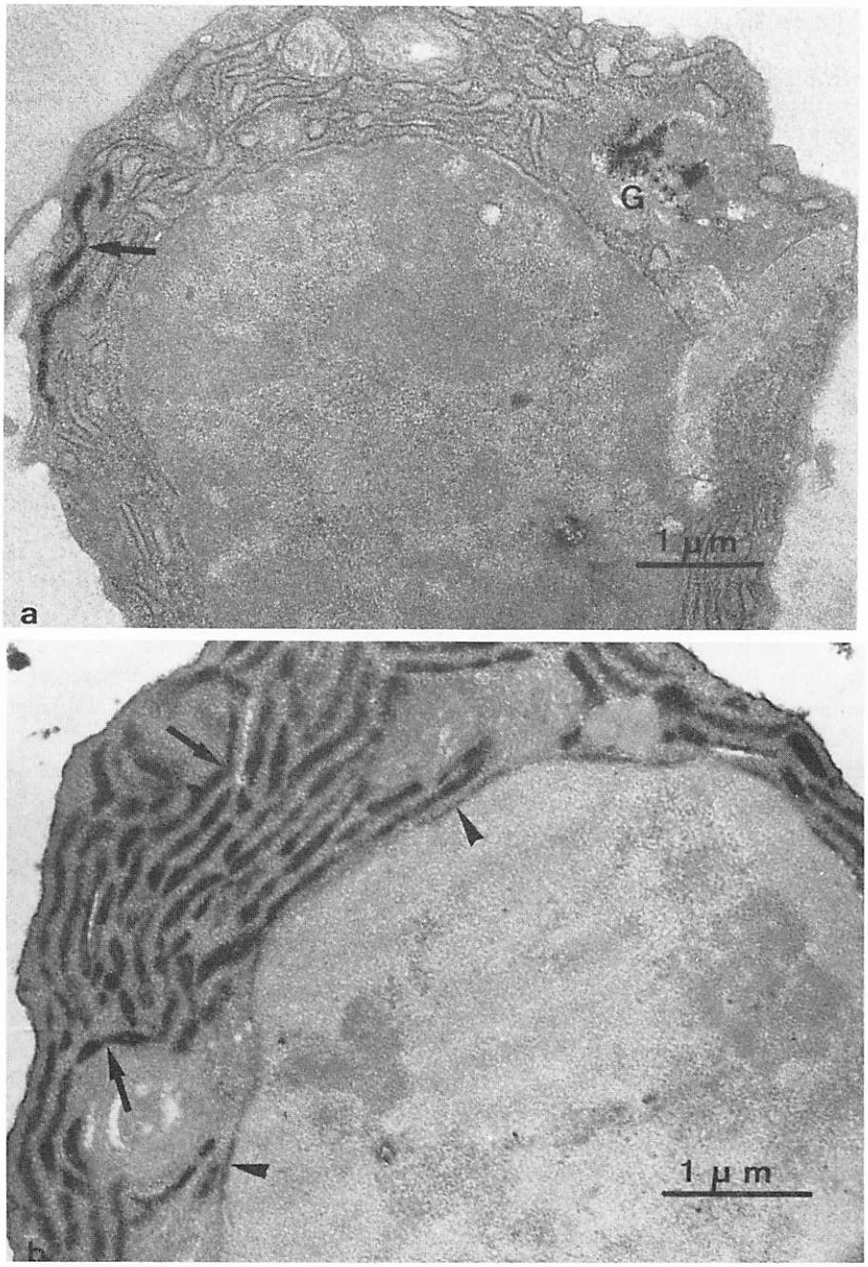


Figure 6-1. (a) Single cell suspension from alkaline phosphatase immunized lymph node and fixed in aldehydes; staining of specific antibodies with alkaline phosphatase (80000 daltons) and substrate. Note enzyme reaction only in few RER lamellae (←) and in the Golgi area (G). Lead salt counterstain. (b) Cryostat section from aldehyde fixed lymph node block; specific staining of IgG in RER (←) and perinuclear space (◀) by use of 7S antibody-HRP conjugates. Lead salt counterstain (from W.D. Kuhlmann, *Prog. Histochem. Cytochem.* **10/1** [1977].1).

cellular ultrastructure is difficult to control. Detergents may lead to damage of RER cisternae with consequent displacement of molecules, and clear-cut immunolocalization with unambiguous ultrastructure is not the rule (Fig. 6-2).

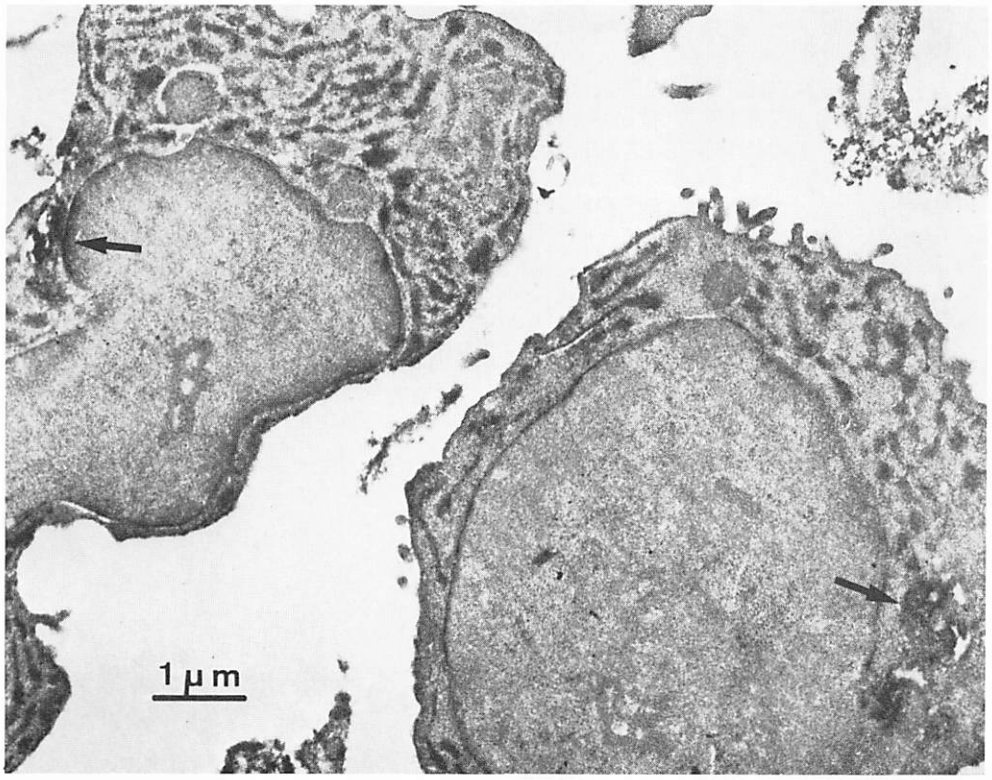


Figure 6-2. Lymph node cell suspension fixed in aldehydes, treated with 0.002 % Tween 80 and reacted with HRP conjugated anti-IgG antibodies. Note fairly-well stained intracellular compartments (RER lamellae and PNS), but diffusion artefacts are also seen (←).

Nevertheless, no convincing results are obtained when whole tissue fragments cut by hand are treated e.g., with saponin (0.1 % w/v saponin) which is either included in the aldehyde fixative and/or in subsequent buffer washings and the HRP labeled antibodies. Instead, only the cells lining the surface of tissue blocks (1 – 2 cell layers thick) will come into contact with conjugates, and most often these are heavily damaged. Such damage can already be expected by the preparation of small tissue fragments with razor blades (Fig. 6-3).

Apart from deficient penetration of enzyme labeled antibodies into aldehyde fixed tissue blocks, at least two further handicaps exist and are due to the fact that the enzyme substrate *per se* diffuses sluggishly:

- Firstly, the substrate does not reach deeper cell layers in the given tissue block (Fig. 6-3d);
- Secondly and very importantly, the substrate will be avidly consumed by the enzyme-conjugate which is immunologically bound to the surface cell layers of the tissue block (Fig. 6-3a-c); AFP is a secreted protein and occurs in both intra- and extracellular spaces.

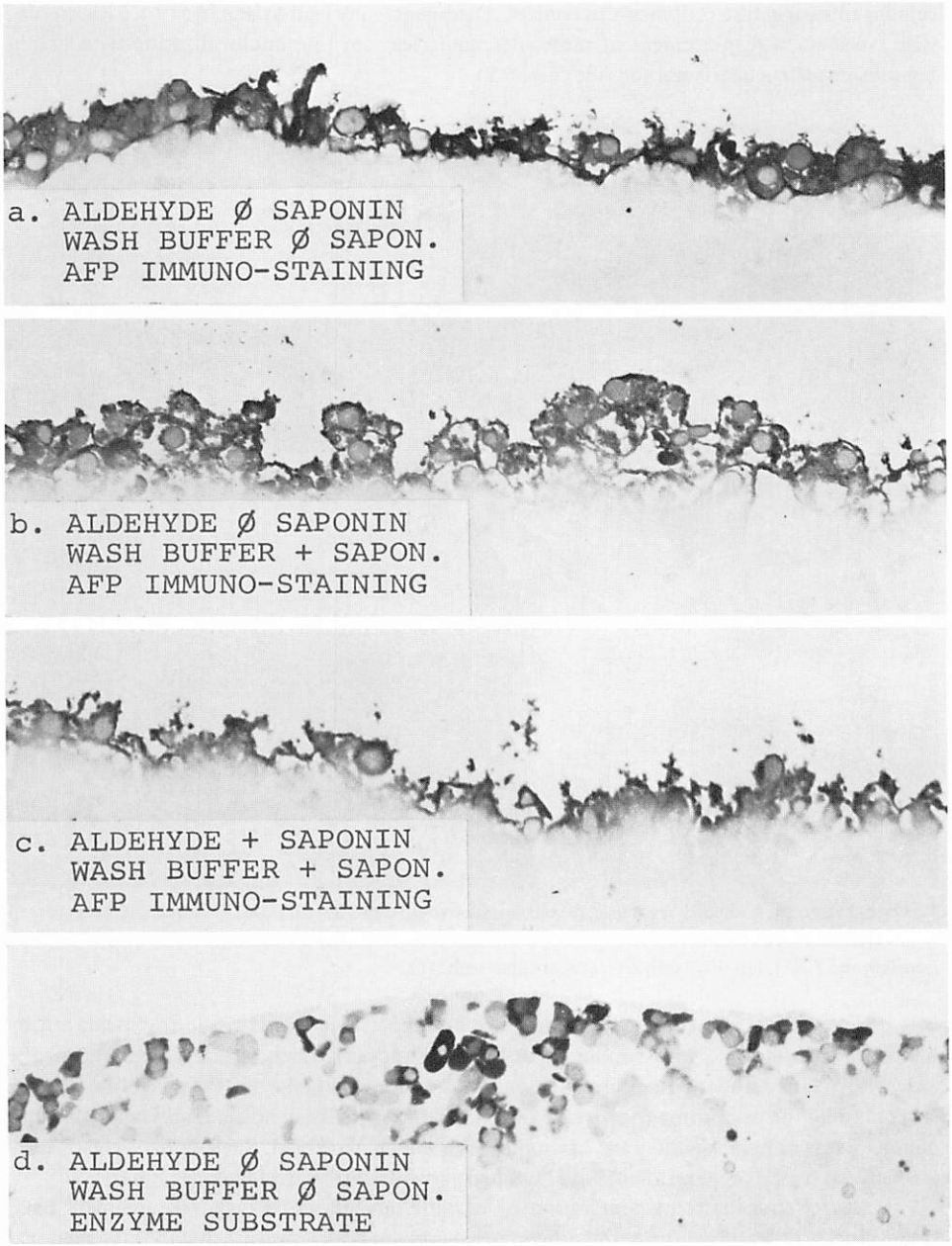


Figure 6-3. Fetal rat liver which is mainly composed of hepatocytes and peroxidase positive hemopoietic cells was employed. Tissue blocks were cut by hand, fixed (with or without saponin), washed in buffer (with or without saponin) and submitted to immuno-staining for AFP (a – c). In one case, conventionally fixed and washed tissue blocks (no saponin) were only stained with enzyme substrate (d). Note the diffusion gradient into tissue blocks of both conjugates and enzyme substrate irrespective of the pretreatments. Semithin sectioning after resin embedment, original × 250.

An experiment which explains the second phenomenon is shown in Fig. 6-4: in (a), we see the typical staining of endogenous peroxidase activity in erythrocytes (aldehyde fixed) because of sufficient penetration of enzyme substrate; in (b), another batch of aldehyde fixed erythrocytes was first reacted with Concanavalin A – peroxidase conjugate (which binds to carbohydrate portions of the cell surface), then stained in the usual way with DAB/H_2O_2 substrate. Here, endogenous peroxidase activity is hardly seen, whereas heavy staining of the erythrocyte surface is obvious; HRP conjugated to Concanavalin A (the latter then bound to the cell surface) has almost completely captured the substrate.

The above penetration studies exemplify briefly the difficulties encountered with antibody conjugates in preembedment immunohistology. In order to overcome penetration problems, thick sections may be prepared with tissue choppers or with cryomicrotomes [1]. Hitherto, the best results have been obtained with thick frozen sections from well-fixed specimens [2, 3, 10, 11]. Indeed, careful fixations are necessary to avoid, as much as possible, diffusion artefacts and cell damage by the freeze-thaw cycles and to manipulate the fragile preparations in the course of immuno-staining. The adoption of cryomicrotomy to preembedment immunohistology is not difficult. A typical protocol of the preparative steps is described in detail. The experimental model shows the intracellular localization of AFP in rat liver.

Protocol of immuno-staining:

1. Tissue sampling: livers are sliced into cubes of about 2 mm and fixed at 0 °C in cacodylate buffered (pH 7.2) 6 % formaldehyde for 5 h followed by 6 % formaldehyde plus 0.25 % glutaraldehyde for 60 – 90 minutes (fixation established by trial). Blocks are then washed overnight in buffer.
2. Pretreatment (Fig. 6-5): tissue blocks are soaked with 10 % dimethylsulfoxide (DMSO)/cacodylate buffer for lh, rapidly frozen in liquid nitrogen cooled isopentane and cut at 10 – 40 μm in a commercial cryostat. Frozen cut sections are dropped straight into small vials containing 10 % DMSO and subsequently washed three times with cacodylate buffer. Sections are kept in these vials until final resin embedment. Prior to incubation in antibodies, endogenous peroxidases can be inhibited by treatment with 1 – 2 % H_2O_2 for 60 minutes. All reactions are carried out at room temperature.
3. Incubation: sections are floated in conjugates (0.5 mg/mL) for at least 2 h; overnight incubation also proves useful. Then, sections are rinsed three times and washed (3 × 10 min) in buffer followed by fixation in 0.5 % glutaraldehyde for 5 min and further washings (3 × 10 min).
4. Controls: specificity is controlled by incubation of parallel sections in (a) HRP labeled IgG globulins from nonimmune rabbit; (b) HRP labeled anti-glucose oxidase antibodies. Sections are subsequently treated as in (3), followed by enzyme substrate.
5. Enzymo-cytochemical detection of HRP: DAB cytochemistry according to Chapter 3.2. After washings in cacodylate buffer, the preparations are postfixed in 1 % OsO_4 for 30 minutes.
6. Dehydration and resin embedment: sections are dehydrated in ascending series of ethanol and flat embedded in Epon [12].

A series of the preparative steps is shown in Fig. 6-6 (from left to right): frozen cut sections are immuno-stained, washed, dehydrated and infiltrated with complete Epon embedding mixture in small glass vials. For light microscopy, some sections are placed with a needle on slides and mounted under coverglass. For electron microscopy, single sections are placed into polyethylene lids (from Beem capsules or equivalent) containing a droplet of fresh Epon mixture. From polymerized Epon molds (prepared in advance in lids of Beem capsules), Epon is removed and put with the flat surface on top of the section. After subsequent polymerization a desired part of the section is selected by light microscopy. Then, tissue and Epon excess are cut away with a hot razor blade. The preparation is mounted with adhesive (or Epon) on a

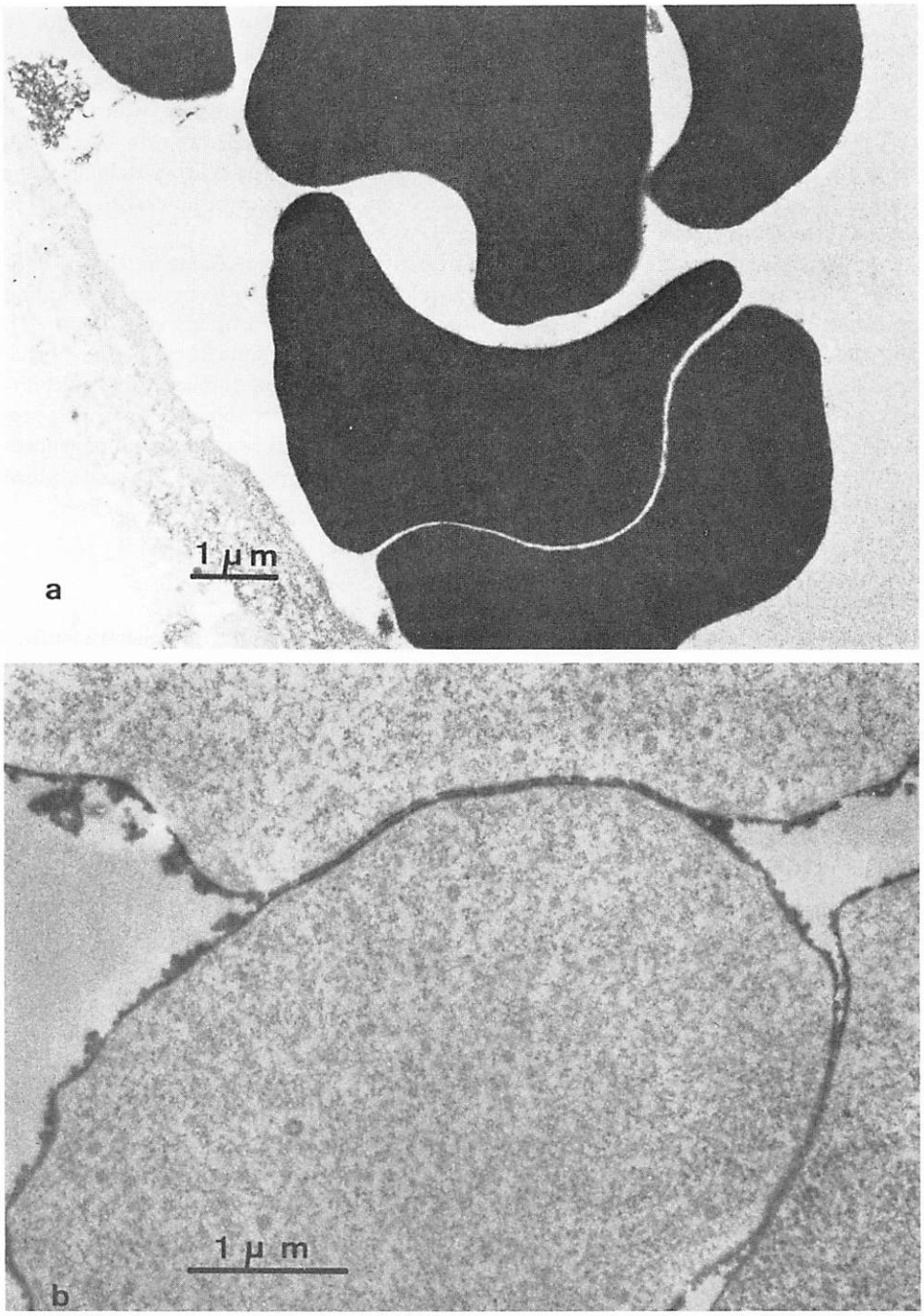


Figure 6-4. Staining of endogenous peroxidases. (a) Aldehyde fixed erythrocytes; (b) aldehyde fixed erythrocytes and reacted with Concanavalin-A-HRP conjugates prior to incubation in DAB/H_2O_2, see text.

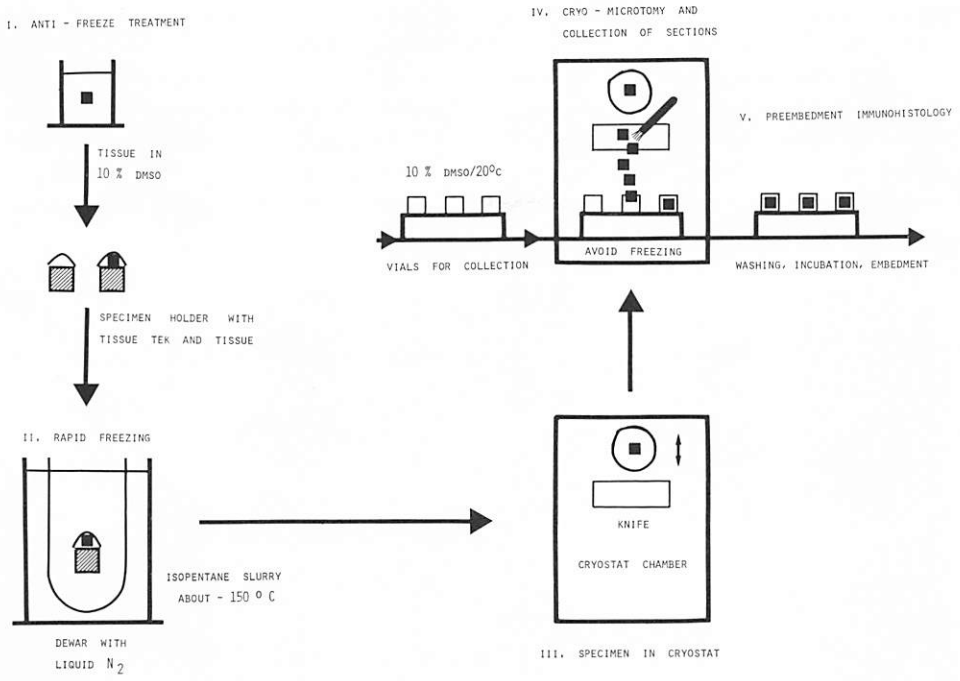


Figure 6-5. Steps of tissue processing for preembedment immunohistology, see text.

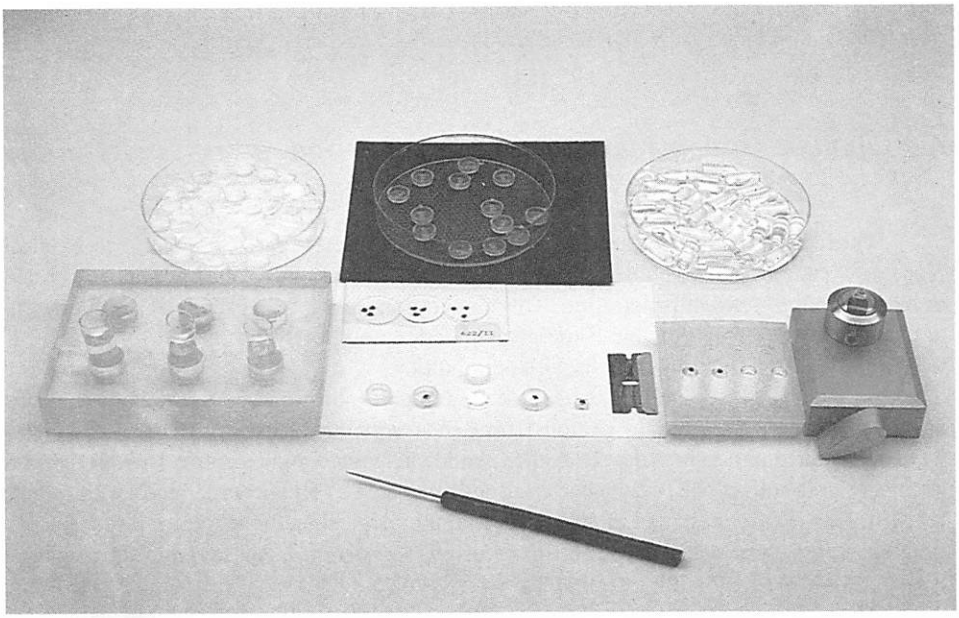


Figure 6-6. Steps of tissue processing from incubation to Epon embedment.

gelatine capsule filled with Epon (and polymerized in advance). Finally, the selected tissue area of the flat embedded section is trimmed for ultramicrotomy.

The immunolocalization of AFP by use of the above given direct incubation method in the preembedment period is shown in Fig. 6-7. This example is from a neonatal liver, and the onco-fetal antigen AFP is detected in the PNS and RER lamellae of hepatocytes. A detailed description of the subcellular distribution of AFP in normal and diseased livers has been presented earlier [11, 13 – 15] and in references cited therein. The usefulness of the employed preembedment technique has also been proved [2, 3, 10].

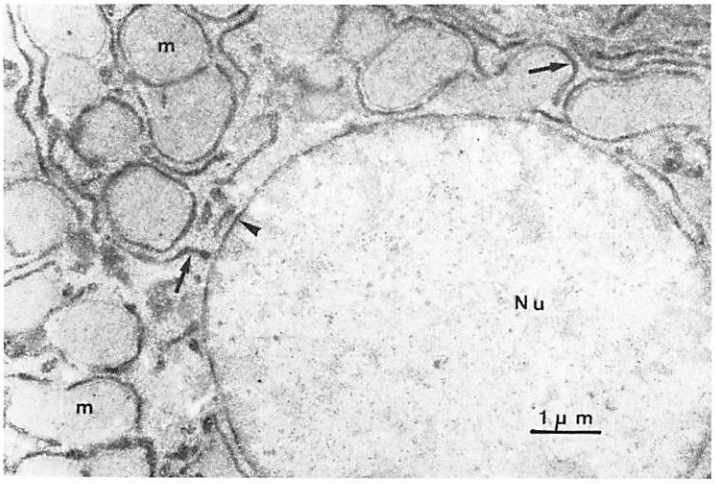


Figure 6-7. Localization of AFP in RER (←) and PNS (◀) of hepatocyte from 3 day-old liver; nucleus (Nu), mitochondria (m) (from W.D. Kuhlmann, *J. Ultrastruct. Res.* **68** [1979] 109).

6.2 *Immuno-staining in Postembedment Period*

The logical way to render intracellular components accessible to immunohistochemical reagents would be to use sections from embedded organs and, thus, labeling can be regarded as independent of reagent penetration. This assumption is correct in principle, but successful antigen staining is only possible if antigenic determinants reach the section surface and if these are still able to bind specifically with antibodies.

Reliable ultrastructural postembedment immunolocalization was occasionally achieved when reagents were applied to sections from epoxy, methacrylate, styrene or BSA cross-linked embedded tissues in order to localize peptide hormones and receptors [16 – 21], bacteriophage/viral antigens [22 – 24], bacterial antigens [25 – 27], pancreatic secretory proteins [28, 29]. However, widespread application of immunological methods to ultrathin resin sections has not yet been achieved. Problems involved in postembedment immuno-staining have been discussed [2, 3, 30 – 32]; see also Chapters 4.3 and 5.3.

The most important factors known to be responsible for loss of immunoreactivity are tissue fixation, dehydration and resin curing (Chapter 4). The extent of all denaturation steps

in tissue embedment is difficult to predict. At all events, we must expect that substantial quantities of antigen reactive sites/regions are no longer reactive in ultrathin sections. Nevertheless important information about loss of antigenicity can be obtained by examination of different schedules of tissue processing and by subsequent immuno-staining of semithin sections according to a technique described by Kuhlmann and Krischan [32]. Thus, we found that sufficient numbers of antigens can remain for subsequent postembedment immunohistology. However, it must be borne in mind that tissue embedment must be adapted to each type of biological specimen.

Despite the numerous pitfalls, the great advantage of immunological ligand assays applied to ultrathin Epon sections has reinforced this kind of investigation [33, 34]. Moreover, recent developments with low-temperature dehydration and embedment in polar resin (e.g., Lowicryl K4M) hold great promise for future postembedment immunohistology [35]. In the meantime, we compared various resin embedments of organs and found that a variety of antigens can be localized with immunoperoxidase methods in semithin sections from Epon 812 embedded organs (see Chapter 5.3). Thus, we became interested in studying this approach with ultrathin sections. We have used rat lymph nodes as model for the intracellular localization of IgG. For a long time, we were not able to stain this protein in ultrathin Epon sections [2, 3], and we were eager to find the reason for this failure. The staining procedure used was an indirect peroxidase labeled antibody method. Various procedures for partial removal of Epon and immunohistological incubations were examined. A typical and finally successful protocol is given in the following.

Protocol of immuno-staining:

1. Tissue sampling: Fixation was established according to principles described in Chapter 4.1. Popliteal lymph nodes are cut into small blocks, fixed in 4 % formaldehyde/cacodylate buffer at 0 – 4 °C for 24 h [3] and washed for several hours by multiple changes of the buffer solution. Blocks are dehydrated with Method A (Table 4 – 2) and embedded in Epon 812 (Table 4 – 3). Ultrathin sections of light gold interference color are cut and collected on Formvar (0.7 %) and carbon-coated 200 mesh Nickel grids.
2. Pretreatment: resin is partially removed with NaOH in ethanol (modified from Lane and Europa [36]). A stock solution (2 g NaOH in 100 mL absolute ethanol) is prepared and diluted 1 : 2 with distilled water; sections are floated at room temperature for 10 min on drops of this solution, passaged on 50 % ethanol followed by distilled water, then placed on 0.2 % BSA/PBS for 5 minutes. All subsequent steps are carried out at room temperature, and treatments are done by floating the grids on droplets of the various solutions on sheets of Parafilm®. Washing steps are three consecutive passages on the respective solutions. After each step, the grid is blotted by touching its edge onto filter paper (do not dry).
3. Incubation: the indirect peroxidase labeled antibody method is described; all sera are diluted in 0.2 % BSA/PBS. The following incubation sequence is chosen:
   - first step, rabbit anti-rat IgG antibodies (0.001 mg/mL) for 8 h;
   - second step, sheep anti-rabbit IgG antibodies (0.01 – 0.1 mg/mL) for 10 min;
   - third step, rabbit anti-sheep IgG antibodies labeled with HRP (0.01 – 0.1 mg/mL) for 10 minutes. The respective antibodies which have not reacted are washed off with 0.2 % BSA/PBS, then grids are fixed with 0.5 % glutaraldehyde/PBS for 1 min and washed with 0.2 mol/L Tris-HCl buffer pH 7.4.
4. Controls: specificity is controlled on serial sections in which the primary antibodies are replaced by (a) normal rabbit IgG globulins; (b) rabbit anti-glucose oxidase antibodies (unrelated antibodies). Grids are then treated as in (3), followed by enzyme substrate. Furthermore, results of postembedment immunoelectron microscopy are compared with results obtainable by preembedment immuno-staining. The latter procedure is exactly the same as that used earlier [2, 3]; for technical details see protocol of immunostaining in Chapter 6.1.
5. Enzymo-cytochemical detection of HRP: 2.5 mg DAB are dissolved in 10 mL Tris-HCl buffer and passed through 0.22 μm filter. 25 μL of 1 % H_2O_2/distilled water are added and the solution is gently

stirred. Grids are held 3 – 5 min into the substrate followed by washings with distilled water. The cytochemical reaction product is intensified by postfixation with 0.1 % OsO_4/distilled water for 10 minutes. Finally, grids are rinsed in distilled water and air-dried. Controls with the preembedment staining technique are reacted as described in Chapter 6.1.

A prerequisite for the present immuno-staining was the partial removal of Epon from ultrathin sections. From above chapters we know that polymerization of Epon forms three-dimensional structures by the formation of cross-links. Yet, only partially reacted compounds can also occur when Epon is cured in steps of increasing temperatures (Chapter 4.3). Unreacted monomers and oligomers are thought to modify fewer antigens in the course of polymerization and these may by readily washed off from sections prior to incubation in antibodies. Apart from this, sodium methoxide in a mixture of methanol and benzene and alcoholic sodium hydroxide will break up at least some of the cross-links (Chapter 5.3). However, this step must be carefully executed in order to obtain sufficient removal of Epon on the one hand and not to destroy the ultrathin section on the other hand. As a rule, this etching procedure dissolves ultrathin sections very rapidly, so that controlled etching is difficult to achieve. Treatment of sections with diluted solutions of alcoholic sodium hydroxide are easier to handle. To this aim, a stock solution of 2 % NaOH in absolute ethanol is diluted 1 : 2 with distilled water. The optimal reaction time in our case is 10 min, whereas treatment for 20 min leads to section damage so that cells become extracted (Fig. 6-8 and Fig. 6-9). Also, 2 %

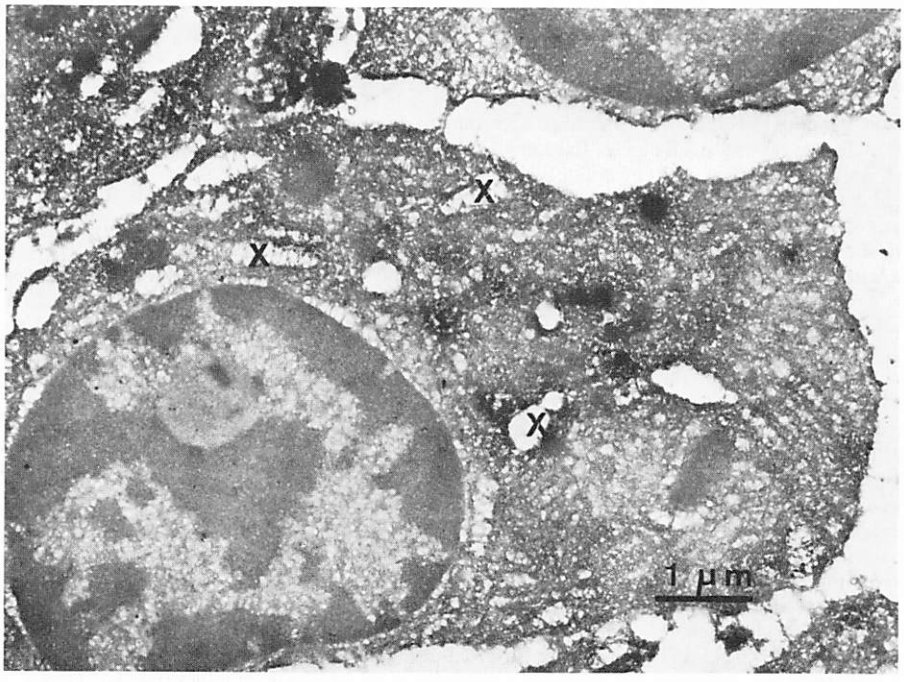


Figure 6-8. Ultrathin section from Epon embedded rat lymph node with treatment in NaOH (final concentration 1 %) in 50 % ethanol for 20 min and followed by immunolocalization of IgG. Note heavy damage of ultrastructural details (x) in all cellular compartments and impaired immunoreactivity. (Figures 6-8, 6-9 and 6-10 from W.D. Kuhlmann and P. Peschke, *Histochemistry* **75** [1982] 151).

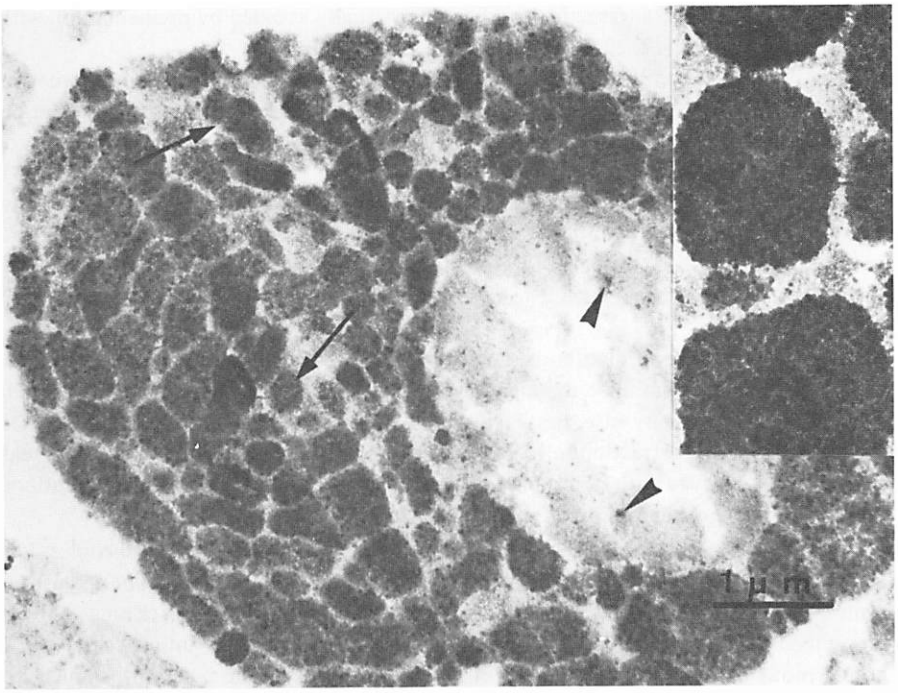


Figure 6-9. Ultrathin Epon section was etched with NaOH (1 %) in a solution of 50 % ethanol as in Fig. 6-8, but treatment was only for 10 min. Strong immuno-staining of IgG is seen in cisternae of the RER (←). Few but negligible background reactions are seen in the nucleus (◀). Inset: higher magnification view of reaction product; note its granular appearance.

NaOH in absolute ethanol or further dilution in absolute ethanol damage ultrastructural details irrespective of the reaction times employed. Detailed descriptions of results and principles for etching of Epon sections were given earlier [32, 33, 36 – 38].

Contrary to publications in which treatment of resin sections with hydrogen peroxide was suggested to be important for subsequent immuno-staining [17, 39], we must state that pretreatment of sections with hydrogen peroxide did not enable immuno-staining of IgG in ultrathin Epon sections. At least for the model examined here we have no evidence that hydrogen peroxide could break up and remove Epon from sections sufficiently. In the case of lysozyme staining in Paneth cells, H_2O_2 etching of sections also had no effect [33]. Conflicting results were described for IgA staining. H_2O_2 treatment was reported to enable immuno-staining at the electron microscopic level, although with considerable impairment of ultrastructural preservation of the cells [34]. On the other hand, no immuno-staining was found with H_2O_2 etching at the light microscopic level [40]. Finally, no H_2O_2 etching at all was necessary for the immunolocalization of secretory proteins in sections of exocrine pancreas by the protein A – gold technique [29]. From all the above it became clear that whether resin etching should be performed or not must be examined for each individual model.

Further treatment of ultrathin sections prior to incubation in antibodies was necessary in order to reduce so-called background staining. The latter is known to be due to nonspecific

sticking of immune sera to sections and can be virtually avoided by preincubation with nonimmune sera or BSA [17, 20, 32, 39].

Antibody concentrations and incubation times were chosen by trial and were within the known limits of immunohistological assays [3, 20, 32, 41]. The minimal incubation time examined for the primary antibodies (rabbit anti-rat IgG) was 30 min and resulted only in faint immuno-staining. Strong reactions were observed after an incubation period of 8 h with a tendency for slight but essentially negligible background staining (Fig. 6-9) which, however, became prominent after overnight incubation. The short incubation times of 10 min each for both second-step (sheep anti-rabbit IgG) and third-step antibodies (HRP labeled rabbit anti-sheep IgG) were chosen because longer incubations did not result in stronger immuno-stainings.

After incubation in antibodies, grids were briefly postfixed with glutaraldehyde. This step was thought to stabilize the antigen-antibody complexes formed, but this was not an essential measure. In any case, best results were obtained with antibodies purified by affinity chromatography. IgG fractions also gave specific reactions, but background can increase. No staining was observed with nonimmune IgG globulins, absorbed and unrelated antibodies in the first step of the immunological incubation series.

The employed DAB/H_2O_2 substrate was adapted from previous schedules described for immunoperoxidase techniques in cryoultramicrotomy [10, 41, 42]. The intensity of enzyme reactions was comparable to that from preembedment immuno-staining. However, in postembedment immunohistology substrate accumulation at the antigen sites was not as homogenous as with the preembedment technique. Instead, the reaction products were of granular appearance for which we have as yet no concrete explanation. The latter became more pronounced on postfixation with OsO_4. The subcellular distribution of IgG by the above postembedment immunohistology corresponded to that obtained with immuno-staining performed in the preembedment phase (Fig. 6-10), but the DAB/H_2O_2 substrate followed by OsO_4 postfixation in the latter usually gave more homogenous and amorphous reaction products than in postembedment immuno-staining.

In both types of experiments (pre- and postembedding localization) immunolocalization was achieved in intracellular compartments described as the rough-surfaced endoplasmic reticulum (RER), the perinuclear space and the Golgi apparatus. However, preembedment immuno-staining resulted in a better ultrastructural preservation of cells than that in post-embedment staining. This fact was readily understood when we realized that preembedment immunolocalization was followed by OsO_4 postfixation prior to ethanol dehydration and Epon embedment. Such double fixation is known to stabilize the ultrastructure of cells significantly better than the single aldehyde fixation (especially formaldehyde fixation and followed by ethanol dehydration and Epon embedment) employed for postembedment immunohistology. Also, etching procedures of the Epon sections will damage further cellular structures.

Even if the postembedment method suffered from both loss of some antigenicity due to denaturation and loss of cellular material due to extraction, this staining method was very important to confirm previous results which were obtained by a preembedding technique [2]. It can be expected that comparison of postembedment and preembedment immunolocalizations of cellular ligands would also be useful in other biological models. For example, interpretations as to whether synthesized proteins fill cisternal cavities (free within the lumen) or are attached to their lumenal side (review at LeBouton and Peters Masse [43]) might be facilitated. From the above evidence and also from experiments with pituitary hormones [44]

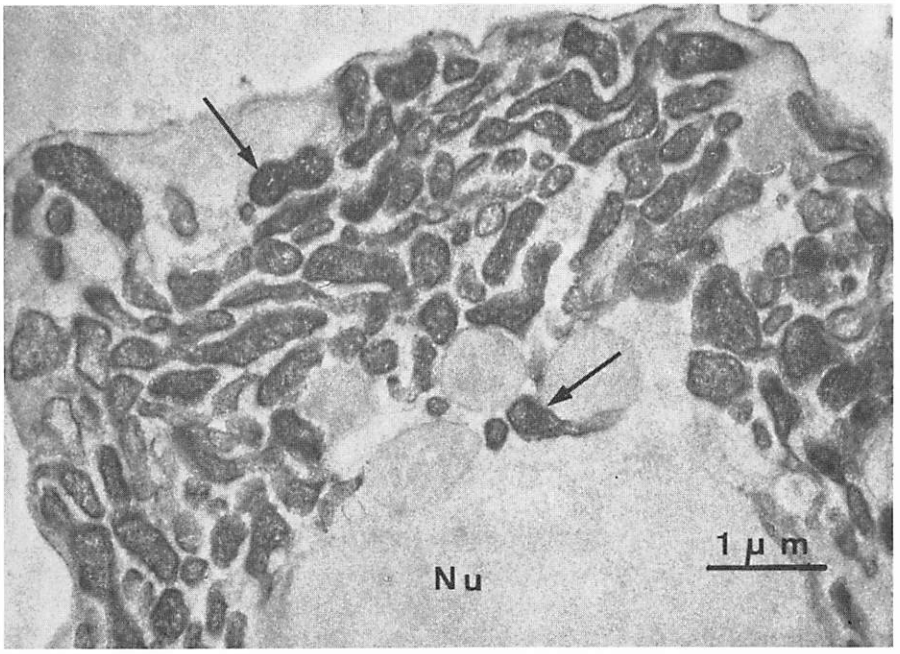


Figure 6-10. Preembedment immuno-staining of IgG in a cryostat section of aldehyde fixed lymph node block. The reaction product in lamellae of the RER (←) appears less granular than that in Fig. 6-9 which is obtained with the postembedment technique; nucleus (Nu).

it is readily understood that preembedment and postembedment immuno-stainings should be applied as complementary techniques in the histological study of organs.

In order to circumvent problems involved with tissue embedment in nonpolar epoxy resin (Epon 812), a polar low-temperature medium (e.g., Lowicryl K4M based on cross-linked acrylate-methacrylate) might be used. The latter embedding medium was developed in order to reduce denaturation and conformational changes usually associated with classical electron microscopic resins [45 – 47]. Lowicryl K4M resin proved suitable for postembedment immunohistology with protein A – gold complex as marker and was suggested to be less deleterious to antigens than Epon inasmuch as specific immuno-staining was enhanced and background labeling was reduced [34]. Low-temperature dehydration and embedment in polar resin are certainly of great interest for future postembedment immunohistology and especially for the localization of very fragile antigens. Also, specific antigen protective measures prior to embedment will be useful (see Chapter 4.4).

6.3 *Immuno-staining in Cryo-ultramicrotomy*

Many cell components can be studied by pre- or postembedment immunohistology. However, one would like to possess an alternative method in order to overcome all problems

observed in pre- or postembedment immuno-staining. Thus, a newer approach which holds great promise is that involving the use of ultrathin frozen sections. Especially for immunohistological work, cryo-ultramicrotomy offers a definite advantage since irreversible modifications of cellular ligands by dehydration and resin embedment and penetration of labeled antibodies is no longer a problem.

Developments of procedures for cryo-ultramicrotomy, suitable for cytochemistry and immunohistological purpose have been published since 1964 [48 – 55]. Several immunohistological procedures including various markers were tried with ultrathin frozen sections [1], but reasonable results were mainly obtained with ferritin; a concise description of the methodology is given by Tokuyasu [55]. Hitherto, direct and indirect peroxidase stainings were most often disappointing inasmuch as the original 3,3'-diaminobenzidine method [56] was very capricious when applied to ultrathin frozen sections, i.e. heavy nonspecific precipitations of oxidized DAB which obscured cellular details [2, 3, 10]. Alternatively, the use of 4-chloro-1-naphthol was proposed as substrate for the immunoperoxidase localization of hormones in pituitary gland [57].

Because of the general interest in peroxidase labeling methods in cryo-ultramicrotomy of organs we searched for further developments of this technique. In subsequent studies, progress was achieved by modification of the immunohistological incubation including DAB staining [41, 42]. Even if combined cryo-ultramicrotomy and immuno-staining is not yet routine, this approach has been successfully applied in research. A typical protocol of this technique is here described. We have used simian adenovirus type 7 (SA_7) infected monkey kidney cells in order to localize virus antigens in the cell nucleus [41].

Protocol of immuno-staining:

1. Tissue sampling: cultivated kidney cells of Cercopithecus aethiops are infected with 10 p.f.u./cell and fixed 62 h later. In a set of experiments, schedules of different fixations were examined. This procedure proves useful: cacodylate buffered 4 % formaldehyde for 10 min followed by 4 % formaldehyde plus 0.25 % glutaraldehyde for 10 min at O°C. Then, cells are harvested from the plastic dish and centrifuged at 5000 rpm for 5 minutes. The obtained pellets are repeatedly washed in buffer, infiltrated with sucrose [54], frozen on a tissue holder by immersing into liquid nitrogen and placed into the cryochamber of a commercial cryo-ultramicrotome. The knife and trough are at -70°C. Sections are cut and spread on 50 % DMSO in distilled water, transferred with a wire loop onto a drop of water at room temperature, then collected on Formvar (0.3 %) and carbon-coated nickel grids and dried overnight at room temperature.
2. Pretreatment: Grids are rehydrated for 1 min on a drop of PBS. All subsequent steps are carried out at room temperature, and treatments are done by floating grids on droplets of the various solutions on sheets of Parafilm®. Washing steps are three consecutive passages on the respective solutions. Grids are not allowed to dry between the steps.
3. Incubation: the indirect peroxidase labeled antibody method is employed. We have no experience with other labeling techniques, e.g. the use of PAP on ultrathin frozen sections;
- 3 % sheep normal serum in PBS for 5 min (sheep/PBS, prevents non-specific adsorptions);
- first step, rabbit anti-adeno type 7 immunoglobulins (0.1 mg/mL) for 5 min followed by washings on sheep/PBS. The employed anti-human adeno 7 immune sera possessed antibody specificities against the bulk of virus antigens, but these immune sera are nevertheless sufficient to demonstrate the feasibility of the immunoperoxidase method in cryo-ultramicrotomy;
- second step, sheep anti-rabbit IgG antibodies labeled with HRP (0.1 mg/mL) for 5 minutes. Grids are washed with sheep/PBS, then with 0.2 mol/L Tris-HCl buffer pH 7.2 – 7.4.

4. Enzymo-cytochemical detection of HRP: same substrate mixture and procedure as described in Chapter 6.2, but the cytochemical reaction product is not intensified by OsO_4.

It must be emphasized that in combining cryo-ultramicrotomy and immunocytochemistry in which incubations are to be performed just in aqueous solutions, the use of organs with well "insolubilized" cell components (done by fixation) is recommended. Otherwise, diffusion artefacts must be expected. This general aspect of tissue sampling was discussed earlier (Chapter 4.1). Hence, in a set of experiments, schedules of different formaldehyde and glutaraldehyde concentrations and various fixation times were examined on SA_7 infected cultures in order to control both immunoreactivity and ultrastructural preservation. The schedule described above proved satisfactory for our purpose.

Apart from appropriate tissue stabilization by fixation prior to cryo-ultramicrotomy, it proved useful to support the cells with an "inert" material. Although ultrathin frozen sections can be obtained without enveloping the tissue, a supporting matrix enables convenient handling in dry sectioning as well as for spreading of sections on a liquid surface of the knife trough. To this aim, gelatin support [51], BSA cross-linked envelopment [53] or sucrose infusion of tissues [54] have been proposed. For detailed discussion of the advantages and disadvantages of those techniques we refer to those publications.

In the above proposed model, pellets of aldehyde fixed monolayer cells were infused with sucrose. This step was especially useful for cell suspensions because in contrast to solid tissue blocks the *in vitro* cultivated cells proved very fragile. Sucrose infusion is known to enhance plasticity of cells and to prevent freezing-damage by a too large ice crystal formation [55].

We usually spread frozen cut sections on DMSO and transfer them to distilled water at room temperature. We have also taken sections directly from the knife on a drop of sucrose solution to carry them outside the cryochamber. The latter technique is more intricate, but is superior to DMSO spreading in the knife trough. Finally, the drying of sections overnight was found to stabilize the ultrastructure of cells [58]. Indeed, the ultrastructure of overnight dried and then immuno-stained cells often showed better cellular conservation than the immediately stained preparations. The antigens studied here were not denatured by that measure.

Antibody concentrations and incubation times were empirically determined by experiments at both light and electron microscopic levels. In earlier publications we have noted difficulties in immunoperoxidase labeling of cell components in ultrathin frozen sections [2, 10]. These difficulties were attributed to the original DAB cytochemistry whose advantage is the high insolubility of the oxidized product and its osmiophilic character, thus, giving excellent electron density. However, DAB staining proved very capricious in cryo-ultramicrotomy and usually gave heavy nonspecific precipitations which obscured cellular details. In subsequent studies, substrate concentrations were diminished, incubation times drastically shortened and, moreover, grids were passaged on serial baths of freshly prepared enzyme substrate. This procedure enhanced staining specificity, so that meaningful progress could be achieved.

Results from immuno-staining of SA_7 viruses in ultrathin frozen sections of infected cells are shown in Fig. 6-11. The cytochemical reaction is strong and confined to the viruses which are specially arranged in a pseudocrystalline form in the inclusion bodies. This distribution corresponds to that which has been described by classical electron microscopy [59]. Uninfected cells do not stain. Also, infected cells which are incubated with unrelated antibodies (anti-glucose oxidase) or with normal rabbit IgG do not stain. It is suggested that the above staining procedure can be adapted to other cell systems.

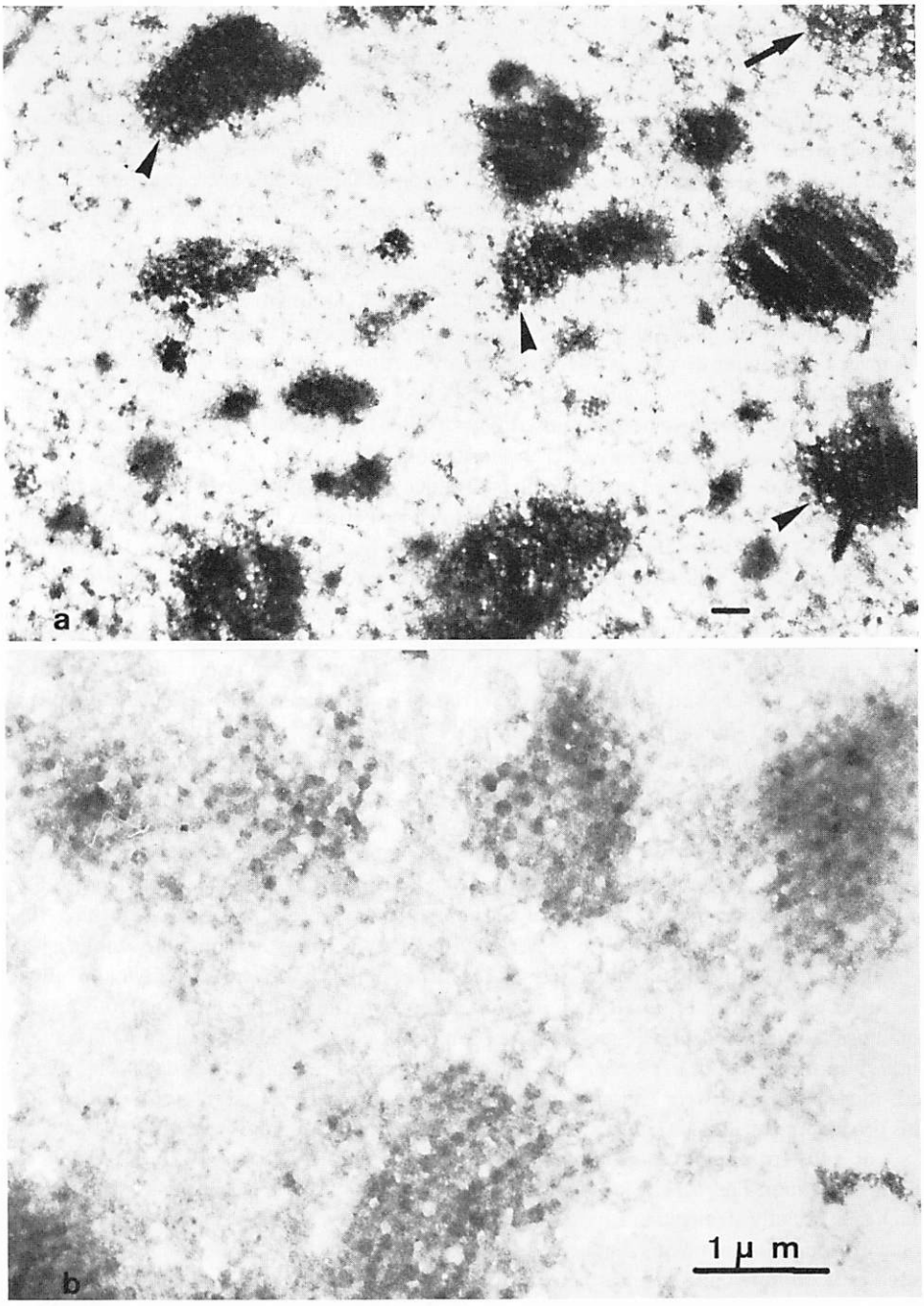


Figure 6-11. Immunoperoxidase stained SA_7 viruses in an infected cell. (a) Note staining of dispersed virus particles at the periphery of the nucleus (←) and viruses in pseudocrystalline form (◀). (b) High magnification view of stained viruses in the inclusion body (from W.D. Kuhlmann and A. Viron, *Biol. Cell* **41** [1981] 153).

6.4 Miscellaneous Techniques

Although we have considered especially the study of organs in their entity by CTEM, this does not mean that immuno-assays either with scanning electron microscopy (SEM) or in combination with freeze-fracture/freeze-etching or dispersive immuno-electron microscopy are not important techniques. Indeed, these approaches contribute significantly to the understanding of biological phenomena even at the molecular level. Because they are part of a highly specialized approach which might be associated with immunohistology in its broadest sense, they will be only mentioned briefly.

The most widely employed marker molecules (cf. also Chapter 2.1) for scanning immuno-electron microscopy have hitherto been latex particles [60, 61], hemocyanin [62], viruses [63] or colloidal gold [64, 65]. In another approach, cathodoluminiscence with fluorescein labeled antibodies was successfully employed for studies at both light microscopic and SEM levels [66]. In fact, the above described principles of antibody preparation hold also true for these purposes, and tissue conservation (see above chapters) must be reconciled with the usual preparative steps in SEM. For details of combined freeze fracture/freeze-etching and immunological ligand assays we refer to Pinto da Silva et al. [67].

Dispersive immuno-electron microscopy may or may not be defined as "immunohistology" in its proper sense because compounds or molecules are taken from their cellular integrity and are studied in their isolated states. Yet, dispersive immuno-electron microscopy is a useful tool for the characterization of antigenic composition of particles, and immunological typing of viruses is one of its applications. Dispersive immuno-electron microscopy dates back to the beginning of electron microscopy when it was observed that a mixture of tobacco viruses and its specific immune serum resulted in aggregation of the virus particles [68, 69]. Later, the introduction of the negative staining technique greatly enhanced contrast [70]. Now, dispersive immuno-electron microscopy is widely used for diagnostic purposes as a method for the detection and characterization of non-cultivable viral agents; for technical details and references see Kapikian et al. [71].

Moreover, dispersive immuno-electron microscopy is an important tool for direct observations of antigen-antibody complexes at the molecular level by use of unlabeled purified antibodies reacted with individual antigens and followed by negative staining, e.g. antibody-hapten complex [72], bacterial flagella [73], ribosomes [74], nucleosides [75]. In cases where negative staining is not successful, high resolution shadowings were reported to enable direct visualization of specific reactions between antibodies and DNA molecules by bright field or dark field electron microscopy [76, 77].

References

1. E.H. Leduc, G.B. Scott and S. Avrameas, "Ultrastructural localization of intracellular immune globulins in plasma cells and lymphoblasts by enzyme-labeled antibodies", *J. Histochem. Cytochem.* 17 (1969) 211.
2. W.D. Kuhlmann, S. Avrameas and T. Ternynck, "A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates", *J. Immunol. Meth* 5 (1974) 33.

3. W.D. Kuhlmann, "Ultrastructural immunoperoxidase cytochemistry", *Prog. Histochem. Cytochem.* **10/1** (1977) 1.
4. S. Yokota and H.D. Fahimi, "Immunocytochemical localization of catalase in rat liver", *J. Histochem. Cytochem.* **29** (1981) 805.
5. S. Dales and P.J. Gomatos, "The uptake and development of Reovirus in strain L cells followed with labeled viral ribonucleic acid and ferritin-antibody conjugates" *Virology* **25** (1965) 193.
6. W. Bohn, "A fixation method for improved antibody penetration in electron microscopical immunoperoxidase studies", *J. Histochem. Cytochem.* **26** (1978) 293.
7. P. Laurila, I. Virtanen, J. Wartiovaara and S. Stenman, "Fluorescent antibodies and lectins stain intracellular structures in fixed cells treated with nonionic detergent", *J. Histochem. Cytochem.* **26** (1978) 251.
8. J.G. Hall, M.S.C. Birbeck, D. Robertson, J. Peppard and E. Orlans, "The use of detergents and immunoperoxidase reagents for the ultrastructural demonstration of internal immunoglobulin in lymph cells", *J. Immunol. Meth.* **19** (1978) 351.
9. I. Ohtsuki, R.M. Manzi, G.E. Palade and J.D. Jamieson, "Entry of macromolecular tracers into cells fixed with low concentrations of aldehydes", *Biol. Cellulaire* **31** (1978) 119.
10. W.D. Kuhlmann and H.R.P. Miller, "A comparative study of the techniques for ultrastructural localization of antienzyme antibodies", *J. Ultrastruct. Res.* **35** (1971) 370.
11. W.D. Kuhlmann, "Ultrastructural detection of alpha$_1$-fetoprotein in hepatomas by use of peroxidase-labeled antibodies", *Int. J. Cancer* **22** (1978) 335.
12. J.H. Luft, "Improvements in epoxy resin embedding methods", *J. Biophys. Biochem. Cytol.* **9** (1961) 409.
13. W.D. Kuhlmann, "Immuno-electron microscopy of α_1-fetoprotein during normal development of rat hepatocytes", *J. Ultrastruct. Res.* **68** (1979), 109.
14. W.D. Kuhlmann, "Alpha-fetoprotein: cellular origin of a biological marker in rat liver under various experimental conditions", *Virchows Arch. (Pathol. Anat.)* **393** (1981) 9.
15. A. Guillouzo, L. Belanger, C. Beaumont, R. Briggs, J.F. Chiu, B. Fleischer, S. Fleischer and J.P. Valet, "Light and electron microscope immunolocalization of alpha-fetoprotein in rat liver cells in vivo and in vitro", *Scand. J. Immunol.* **8** suppl. 8 (1978) 289.
16. P.K. Nakane, "Classifications of anterior pituitary cell types with immunoenzyme histochemistry", *J. Histochem. Cytochem.* **18** (1970) 9.
17. G.C. Moriarty, "Adenohypophysis: ultrastructural cytochemistry. A review", *J. Histochem. Cytochem.* **21** (1973) 855.
18. L.A. Sternberger and J.P. Petrali, "Quantitative immunocytochemistry of pituitary receptors for luteinizing hormone-releasing hormone", *Cell Tiss. Res.* **162**, (1975) 141.
19. R. Martin, E. Weber and K.H. Voigt, "Localization of corticotropin- and endorphin-related peptides in the intermediate lobe of the rat pituitary", *Cell Tiss. Res.* **196** (1979) 307.
20. L.A. Sternberger: *Immunocytochemistry*. J. Wiley & Sons, New York 1979.
21. G. Pelletier, R. Puviani, O. Bosler and L. Descarries, "Immunocytochemical detection of peptides in osmicated and plastic-embedded tissue", *J. Histochem. Cytochem.* **29** (1981) 759.
22. G.E. Striker, E.J. Donati, J.P. Petrali and L.A. Sternberger, "Post-embedding staining for electron microscopy with ferritin-antibody conjugates", *Exp. Mol. Pathol.* suppl. **3** (1966) 52.
23. J.D. McLean and S.J. Singer, "A general method for the specific staining of intracellular antigens with ferritin-antibody conjugates", *Proc. Natl. Acad. Sci. (USA)* **65** (1970) 122.
24. M.S. Shahrabadi and T. Yamamoto, "A method for staining intracellular antigens in thin sections with ferritin-labeled antibody", *J. Cell. Biol.* **50** (1971) 246.
25. L.A. Sternberger, E.J. Donati, J.J. Cuculis and J.P. Petrali, "Indirect immunouranium technique for staining of embedded antigen in electron microscopy", *Exp. Mol. Pathol.* **4** (1965) 112.
26. R.O. Thomson, P.D. Walker, I. Batty and A. Baillie, "Post-embedding staining with ferritin labeled antibodies", *Nature (Lond).* **215** (1967) 393.
27. H. Takamiya, S. Batsford, H. Gelderblom and A. Vogt, "Immunoelectron microscopic localization of lipopolysaccharide antigens on ultrathin sections of Salmonella typhimurium", *J. Bacteriol.* **140** (1979) 261.
28. J. Roth, M. Bendayan and L. Orci, "Ultrastructural localization of intracellular antigens by the use of protein A-gold complex", *J. Histochem. Cytochem.* **26** (1978) 1074.

29. M. Bendayan, J. Roth, A. Perrelet and L. Orci, "Quantitative immunocytochemical localization of pancreatic secretory proteins in subcellular compartments of the rat acinar cell", *J. Histochem. Cytochem.* **28** (1980) 149.
30. A. Vogt, H. Takamiya and W.A. Kim: "Some problems involved in postembedding staining". In: G. Feldmann, P. Druet, J. Bignon and S. Avrameas (eds.): *Immunoenzymatic Techniques*. North-Holland and American Elsevier, Amsterdam-Oxford-New York 1976.
31. H. Takamiya, S. Batsford and A. Vogt, "An approach to postembedding staining of protein (immunoglobulin) antigen embedded in plastic: prerequisites and limitations", *J. Histochem. Cytochem.* **28** (1980) 1041.
32. W.D. Kuhlmann and R. Krischan, "Resin embedment of organs and postembedment localization of antigens by immunoperoxidase methods", *Histochemistry* **72** (1981) 377.
33. S.L. Erlandsen, J.A. Parsons and C.B. Rodning, "Technical parameters of immunostaining of osmicated tissue in epoxy sections", *J. Histochem. Cytochem.* **27** (1979) 1286.
34. C.B. Rodning, S.L. Erlandsen and I.D. Wilson, "Immunohistochemical identification of immunoglobulin A on ultrathin tissue sections", *Am. J. Anatomy* **157** (1980) 221.
35. J. Roth, M. Bendayan, E. Carlemalm, W. Villiger and M. Garavito, "Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue", *J. Histochem. Cytochem.* **29** (1981) 663.
36. B.P. Lane and D.L. Europa, "Differential staining of ultrathin sections of Epon-embedded tissues for light microscopy", *J. Histochem. Cytochem.* **13** (1965) 579.
37. H.D. Mayor, J.C. Hampton and B. Rosario, "A simple method for removing the resin from epoxy-embedded tissue", *J. Biophys. Biochem. Cytol.* **9** (1961) 909.
38. W.D. Kuhlmann and P. Peschke, "Advances in ultrastructural postembedment localization of antigens in Epon sections with peroxidase labeled antibodies", *Histochemistry* **75** (1982) 151.
39. P.K. Nakane, "Application of peroxidase-labeled antibodies to the intracellular localization of hormones", *Acta Endocrinologica* (Kopenhagen) suppl. **153** (1971) 190.
40. C.B. Rodning, S.L. Erlandsen, H.D. Coulter and I.D. Wilson, "Immunohistochemical localization of IgA antigens in sections embedded in epoxy resin", *J. Histochem. Cytochem.* **28** (1980) 199.
41. W.D. Kuhlmann and A. Viron, "Immunoperoxidase technique in cryoultramicrotomy for the detection of simian adenovirus in cell nuclei", *Biol. Cell* **41** (1981) 153.
42. W.D. Kuhlmann and A. Viron, "Diaminobenzidine cytochemistry in cryoultramicrotomy for the detection of peroxidases", *Histochemistry* **54** (1977) 331.
43. A.V. LeBouton and J. Peters Masse, "Ultrastructural immunocytochemistry of nascent albumin topology: proposed cytosolic folding and membrane transit of the protein", *Anat. Rec.* **201** (1981) 203.
44. C. Tougard, R. Picart and A. Tixier-Vidal, "Immunocytochemical localization of glycoprotein hormones in the rat anterior pituitary. A light and electron microscope study using antisera against rat β subunits: a comparison between preembedding and postembedding methods", *J. Histochem. Cytochem.* **28** (1980) 101.
45. E. Kellenberger, "Progress and new approaches in obtaining finer significant details in the electron microscopy of biological material", *J. Electron Microsc.* **28** suppl. (1979) 49.
46. E. Kellenberger, E. Carlemalm, W. Villiger, J. Roth and R.M. Garavito: *Low Denaturation Embedding for Electron Microscopy of Thin Sections*. Chem. Werke Lowi, Waldkraiburg 1980.
47. E. Carlemalm, W. Villiger and J.D. Acetarin, "Advances in specimen preparation for electron microscopy. I. Novel low-temperature embedding resins and a reformulated Vestopal", *Experientia* **36** (1980) 740.
48. W. Bernhard and M.T. Nancy, "Coupes à congélation ultrafines de tissu inclus dans la gélatine", *J. Microscopie* **3** (1964) 579.
49. F.L. Dollhopf, G. Werner and E. Morgenstern, "Die Shandon-Reichert-Kühleinrichtung zum Herstellen von Ultradünn- und Feinschnitten bei extrem niederen Temperaturen. II. Anwendungsbeispiele aus Biologie und Technik", *Mikroskopie* **25** (1969) 33.
50. L. Kolemainen-Seveus, "Frozen ultrathin sections", *Proc. 7th Int. Congr. Electron Microsc.* **1** (1970) 432.
51. W. Bernhard and A. Viron, "Improved techniques for the preparation of ultrathin frozen sections", *J. Cell Biol.* **49** (1971) 731.

52. J.R. Iglesias, R. Bernier and R. Simard, "Ultracryotomy: a routine procedure", *J. Ultrastruct. Res.* **36** (1971) 271.
53. W.D. Kuhlmann and A. Viron, "Cross-linked albumin as supporting matrix in ultrathin cryo microtomy", *J. Ultrastruct. Res.* **41** (1972) 385.
54. K.T. Tokuyasu, "A technique for ultracryotomy of cell suspensions and tissues", *J. Cell Biol.* **57** (1973) 551.
55. K.T. Tokuyasu, "Immunochemistry on ultrathin frozen sections", *Histochem. J.* **12** (1980) 381.
56. R.C. Graham and M.J. Karnovsky, "The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique", *J. Histochem. Cytochem.* **14** (1966) 291.
57. G. Morel, M.G. Forest and P.M. Dubois, "Spécificité tissulaire de la fixation de testostérone: mise en évidence par immunocytochimie ultrastructurale après cryoultramicrotomie", *C.R. Acad. Sci. (Paris)* **288** (1979) 1667.
58. E. Puvion and W. Bernhard, "Ribonucleoprotein components in liver cell nuclei visualized by cryoultramicrotomy", *J. Cell Biol.* **67** (1975) 200.
59. G. Moyne, E. Pichard and W. Bernhard, "Localization of Simian Adenovirus 7 (SA_7) transcription and replication in lytic infection. An ultracytochemical and autoradiographical study", *J. Gen. Virol.* **40** (1978) 77.
60. A.F. LoBuglio, J.J. Rinehart and S.P. Balcerzak, "A new immunologic marker for scanning electron microscopy", *Proc. Fifth Ann. Scanning Electron Microscopy* **2** (1972) 313.
61. R.S. Molday, W.J. Dreyer, A. Rembaum and S.P.S. Yen, "Latex spheres as markers for studies of cell surface receptors by scanning electron microscopy", *Nature (Lond.)* **249** (1974) 81.
62. N.K. Weller, "Visualization of concanavalin A-binding sites with scanning electron microscopy", *J. Cell Biol.* **63** (1974) 699.
63. M.F. Lipscomb, K.V. Holmes, E.S. Vitteta, U. Hämmerling and J.W. Uhr, "Cell surface immunoglobulin. XII. Localization of immunoglobulin on murine lymphocytes by scanning immunoelectronmicroscopy", *Eur. J. Immunol.* **5** (1975) 255.
64. M. Horisberger, J. Rosset and H. Bauer, "Colloidal gold granules as markers for cell surface receptors in the scanning electron microscope", *Experientia* **31** (1975) 1147.
65. M. Horisberger and J. Rosset, "Colloidal gold, a useful marker for transmission and scanning electron microscopy", *J. Histochem. Cytochem.* **25** (1977) 295.
66. S.L. Soni, V.I. Kalnins and G.H. Haggis, "Localisation of caps on mouse B lymphocytes by scanning electron microscopy'-, *Nature (Lond.)* **255** (1975) 717.
67. P. Pinto da Silva, S.D. Douglas and D. Branton, "Localization of A antigen sites on human erythrocyte ghosts", *Nature (Lond.)* **232** (1971) 194.
68. M. von Ardenne, H. Friedrich-Freksa and G. Schramm, Elektronenmikroskopische Untersuchung der Präcipitinreaktion von Tabakmosaikvirus mit Kaninchenantiserum", *Arch. Ges. Virusforsch.* **2** (1941) 80.
69. T.F. Anderson and W.M. Stanley, "A study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum", *J. Biol. Chem.* **139** (1941) 339.
70. S. Brenner and R.W. Horne, "A negative staining method for high resolution electron microscopy of viruses", *Biochim. Biophys. Acta* **34** (1959) 103.
71. A.Z. Kapikian, J.L. Dienstag and R.H. Purcell: "Immune electron microscopy as a method for the detection, identification, and characterization of agents not cultivable in an in vitro system". In: N.R. Rose and H. Friedmann (eds.): *Manual of Clinical Immunology.* Am. Soc. Microbiol., Washington D.C. 1976.
72. R.C. Valentine and N.M. Green, "Electron microscopy of an antibody-hapten complex", *J. Mol. Biol.* **27** (1967) 615.
73. A. Feinstein, E.A. Munn and N.E. Richardson, "The three-dimensional conformation of γM and γA globulin molecules", *Ann. N. Y. Acad. Sci.* **190** (1971) 104.
74. I.N. Shatsky, L.V. Mochalova, M.S. Kojouharova, A.A. Bogdanov and V.D. Vasiliev, "Localization of the 3'end of Escherichia coli 16S RNA by electron microscopy of antibody-labeled subunits", *J. Mol. Biol.* **133** (1979) 501.
75. R.J. Frink, D. Eisenberg and D.G. Glitz, "Localization of the site of adenylylation of glutamine synthetase by electron microscopy of an enzyme-antibody complex", *Proc. Natl. Acad. Sci. (USA)* **75** (1978) 5778.

76. G. de Murcia, M.C.E. Lang, A.M. Freund, R.P.P. Fuchs, M.P. Daune, E. Sage and M. Leng, "Electron microscopic visualization of N-acetoxy-N-2-acetylaminofluorene binding sites in ColE1 DNA by means of specific antibodies", *Proc. Natl. Acad. Sci. (USA)* **76** (1979) 6076.
77. E. Nahon-Merlin, E. Delain, D. Coulaud and F. Lacour, "Electron microscopy of the reactions of anti-poly A. poly U and anti-poly I. poly C antibodies with synthetic polynucleotide complexes and natural nucleic acids", *Nucleic Acids Res.* **8** (1980) 1805.

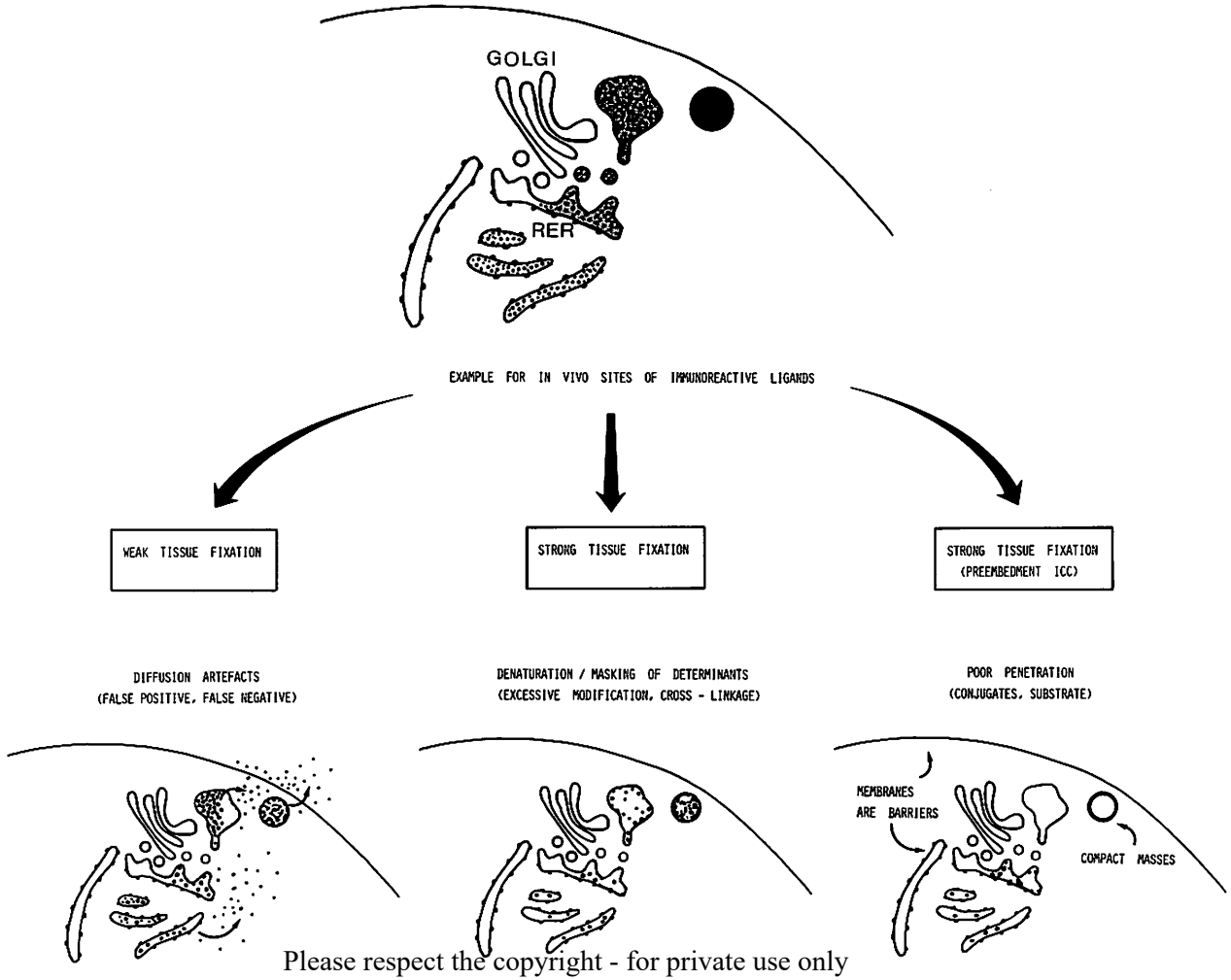


Figure 7-1. Possible roles of fixation in immunohistology, see text.

(a) Endogenous enzyme activity: in the case of enzymes as markers, endogenous enzyme activities in cells will interfere with immuno-staining. For HRP• labeling purposes, endogenous peroxidases can be inhibited by preincubation of tissue preparations with 1 – 2 % H_2O_2 in PBS for periods to be determined experimentally [1].

(b) Non-specific adsorptions: In immunofluorescent work, non-immunological protein-protein interactions were attributed to pH and ionic environment in the incubation media [2]. Comparable non-immunological interactions between tissue components or resin matrix (which is only partially removed in sections from plastic embedded tissue) with whole immune sera can be also observed in immunoenzyme techniques. It is a fact that non-specific adsorptions can be drastically reduced by the use of isolated specific antibodies instead of whole immune sera. Moreover, pretreatment of sections with normal non-immune serum of the species which provides the labeled antibodies leads to a decrease of the section's overall staining. The inclusion of increased concentrations of NaCl (e.g. 0.5 mol/L) and the addition of BSA to the classical PBS buffer wash also leads to significant reduction of non-specific background reactions [3]. This measure may also help in the washing-off of low titered unwanted/cross-reacting antibodies present in the primary immune serum. Last but not lest, the marker molecule itself can be the reason for non-specific staining. Thus, impure HRP preparations were found to give important background reactions [4], and the purest available HRP (RZ 3) is to be preferred.

(c) Staining by unwanted and cross-reacting antibodies: contamination of immune sera by unwanted antibodies and by cross-reacting antibodies will lead to positive staining patterns which are difficult to interprete. Hence, these antibodies must be completely absorbed out of the immune serum prior to its application. The preparation of an immune serum and its specificity must be submitted to careful controls. For immunohistological work one must assure that non-immune IgG, antibodies absorbed with homologous antigens, unrelated antibodies and their conjugates do not give tissue stainings.

(d) Diffusion of antigens: false positive staining can occur by diffusion of molecules away from their original sites when tissues are not sufficiently fixed (see above). Apart from fixation, false positive stainings are also seen when, prior to fixation, cells take up molecules from extracellular spaces (e.g. from serum). Such a passive uptake occurs in damaged/necrotic cells. All these staining reactions are specific from immunological but non-specific from histological points of view and are called ectopic. Passive uptake of molecules can be distinguished by double stainings in one section or by staining of serial sections: first, the antigen under investigation is localized; second, molecules which are normally outside the studied cells are stained. In the case of AFP localization, e.g. in hepatocellular carcinoma, we control the passive uptake of AFP by reaction of one tissue section for AFP and the subsequent second section for serum IgG. We know that the latter is not synthesized by hepatocytes and hepatoma cells [5]. Tissue sites in which both AFP and IgG positivities occur are not taken into account (Fig. 7-2). In consideration of these observations, it is readily understood that immunohistology with organs from necropsy needs rigorous controls.

(e) Enzyme substrate diffusion: generally, enzymes with high specific activity and turnover number and which retain substantial parts of their activity after conjugation with antibodies

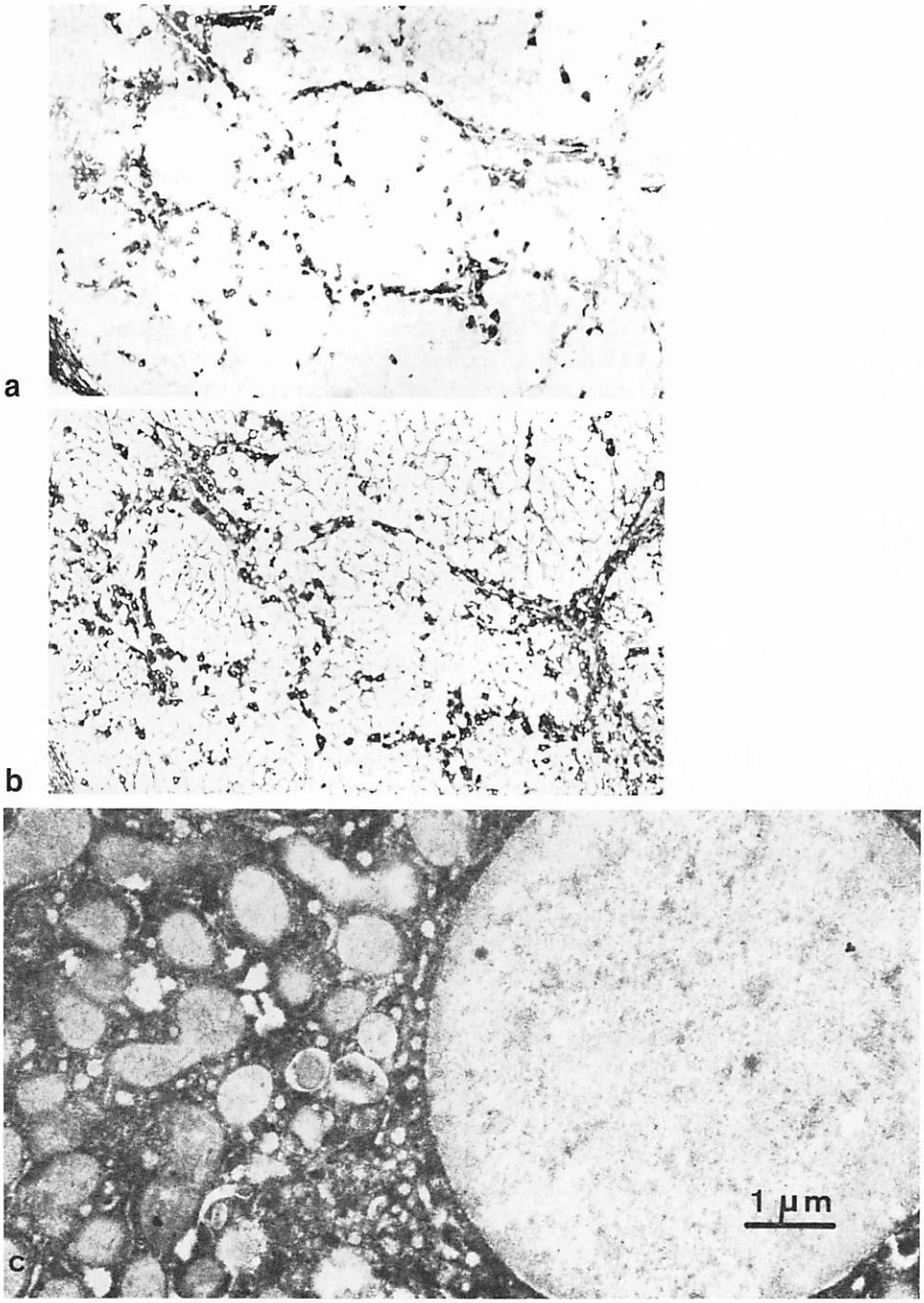


Figure 7-2. Sections through the non-malignant part of a rat liver adjacent to an AFP producing hepatoma; paraffin technique, serial sections. Note leakage of proteins into hepatocytes. (a) Staining for AFP, original × 160. (b) staining for IgG, original × 160. (c) Artefactual AFP staining at the ultrastructural level; diffuse reactions in the cytoplasm and in parts of its organelles.

are preferred. With reduced enzyme activity the formation of the reaction products in cytological assays may be too slow to remain at the enzyme site, thus causing diffusion artefacts. Moreover, enzyme substrates must be employed which give highly insoluble reaction products. In the case of HRP, the DAB cytochemistry proved to be very valuable since by oxidation of DAB with subsequent polymerization and cyclization an extremely insoluble and osmiophilic product is generated [6, 7]; for artefactual stainings by use of DAB see references [8, 9].

7.2 *False Negative Staining*

The main reason for negative results is that tissue fixation was too strong and has led to denaturation of the antigens (see above). However, no fixation or one that is too weak can provoke diffusion of molecules as described above. Thus and apart from false positive immuno-stainings one will also obtain false negative reactions (Fig. 7 – 3). Furthermore, false results occur, too, when conjugates and enzyme substrate do not penetrate organ preparations (preembedment immuno-staining). In the case of postembedment immunohistology,

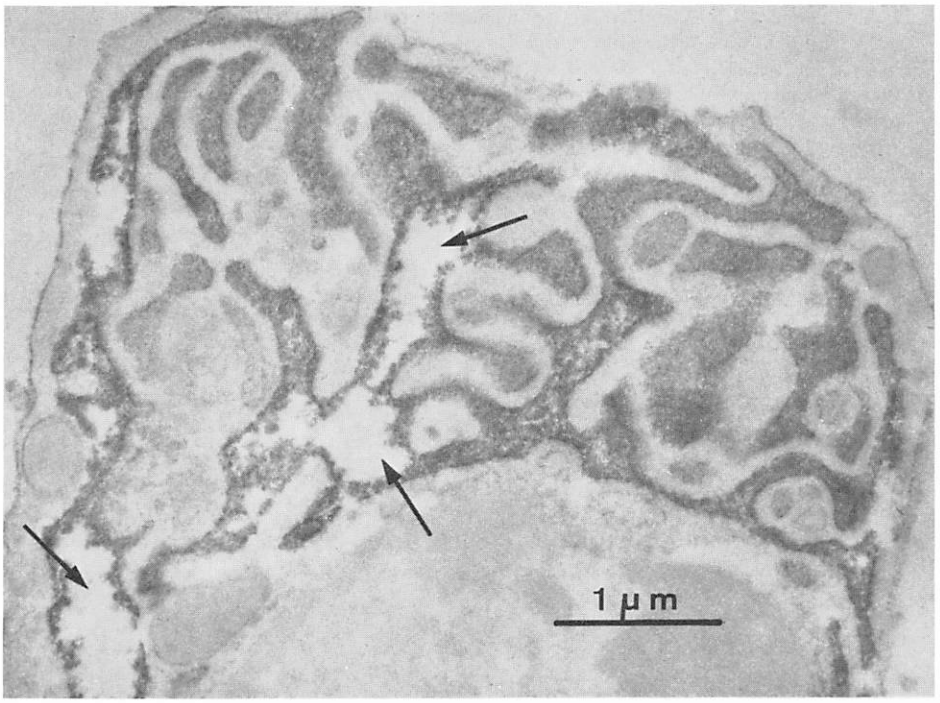


Figure 7-3. Immuno-staining of IgG in a plasma cell; lymph node was fixed in 4 % formaldehyde for 4 h. Note insufficient ultrastructural conservation and false negative staining in RER (←) due to loss of IgG by diffusion.

dehydration by solvents and embedment (e.g. Epon) can also lead to denaturation of antigens. Phenomena of denaturation due to diverse tissue preparations have been discussed in detail (Chapters 4 and 6) and need no repetition.

Finally, we can state that we do not possess and perhaps we shall never have ready-for-use immunohistological schedules for all antigens. What can be done, however, is to adapt procedures to a given problem in order to get information on cellular function in connection with its morphology.

References

1. W.D. Kuhlmann, "Purification of mouse alpha$_1$-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization", *Histochemistry* **44** (1975) 155.
2. C.E. Grossi and H. von Mayersbach, "Technical factors affecting immunohistological methods. I. The influence of different pH and buffer salts for unspecific reactions", *Acta Histochem.* **19** (1964) 382.
3. W.D. Kuhlmann, "Localization of alpha$_1$-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats", *Int. J. Cancer* **21** (1978) 368.
4. W.D. Kuhlmann, "Ultrastructural immunoperoxidase cytochemistry", *Prog. Histochem. Cytochem.* **10/1** (1977) 1.
5. L.L. Miller and W.E. Bale, "Synthesis of all plasma protein fractions except gamma globulins by the liver. The use of zone electrophoresis and lysine-ϵ-C^{14} to define the plasma proteins synthesized by the isolated perfused liver", *J. Exp. Med.* **99** (1954) 125.
6. R.C. Graham and M.J. Karnovsky, "The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique", *J. Histochem. Cytochem.* **14** (1966) 291.
7. A.M. Seligman, M.J. Karnovsky, H.L. Wasserkrug and J.S. Hanker, "Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagents, diaminobenzidine (DAB)", *J. Cell Biol.* **38** (1972) 1.
8. A.B. Novikoff, P.M. Novikoff, N. Quintana and C. Davis, "Diffusion artifacts in 3,3'-diaminobenzidine cytochemistry", *J. Histochem. Cytochem.* **20** (1972) 745.
9. A.B. Novikoff, "DAB cytochemistry: artifact problems in its current uses", *J. Histochem. Cytochem.* **28** (1980) 1036.

8 *Practical Aspects*

The principles and pertinent references of a variety of methods have been described already in the respective chapters. Here, we give in more detail further procedures and formulas which have proved to be useful in this area.

8.1 Polyacrylamide Gel Electrophoresis

A series of stock solutions is prepared in advance with which polyacrylamide (PAA) gels for both analytical and preparative purposes are cast.

Prepare the solutions:

solution I	acrylamide	96.0	g
	BIS	3.4	g
	made up with distilled water to	200	mL
solution II	ammonium persulfate	0.21	g
	made up with distilled water to	100	mL
solution III	ammonium persulfate	0.07	g
	made up with distilled water to	100	mL
solution IV	Tris	36.6	g
	1 mol/L citric acid	28.0	mL
	TEMED	0.48	mL
	made up with distilled water to	200	mL
solution V	solution IV	50.0	mL
	distilled water	50.0	mL
	TEMED	0.05	mL
solution VI	solution IV	10.0	mL
	TEMED	0.16	mL
	made up with distilled water to	100	mL
solution VII	Tris	31.0	g
	boric acid	4.37	g
	made up with distilled water to	125	mL

Gel slab for electrophoresis

An example for the preparation of a PAA gel slab (flat gel for analytical run) in a separating unit of the DESAGA system "HAVANA" is described.

Separation gel

A separation gel with 8 % acrylamide is prepared by mixing the following:

solution I	1.6 mL
solution III	4.8 mL
solution V	2.4 mL
distilled water	0.8 mL

Stacking gel

A stacking gel with 3 % acrylamide is prepared by mixing the following:

solution I	1.0 mL
solution II	8.0 mL
solution VI	4.0 mL
distilled water	3.0 mL

The liquid gel solutions are degassed and slabs are prepared in upright positioned glass chambers. Separation gel is filled into the precooled (4°C) separation chamber and the stacking gel is carefully layered on top; the slot-former is placed into the latter. Polymerization takes about 20 min. In the case of preparative electrophoresis, cathodic and anodic junctions are cast as described (Chapter 1.2.4). For analytical electrophoresis in the DESAGA "HAVANA" system, cathodic and anodic junctions are assured by direct contact of the gel with the respective electrode buffer containers.

Sample preparation

Prior to electrophoresis, samples are desalted (e.g. by gel filtration) and mixed with ethylene glycol 9:1 (vol./vol.). The latter contains a small amount of bromophenol blue as tracking dye. The slot-former is removed, pockets are filled with fresh stacking gel, and samples are then placed underneath with the help of a syringe. After polymerization, the chambers are subjected to electrophoresis.

Electrophoretic run

Electrode buffers are 0.065 mol/L Tris-boric acid at pH 9.0 which are prepared by mixing the following:

solution VII	62.5 mL
made up with distilled water to	2000 mL

Electrophoresis is started with 30 – 40 mA (about 180 V) until samples reach the separation gel (indicated by the tracking dye). Power is then increased and maintained at 50 mA until the tracking dye has passed the whole separation distance; at the end of an electrophoretic rund a potential of about 350 V is recorded. Preparative electrophoretic gel slabs are cut into strips of 3 mm each on a standard cutting device and eluted (Chapter 1.2.4). Analytical PAA gel slabs are fixed and stained.

Fixation and staining of PAA gels

Fixation

Coomassie Brilliant Blue stain: 12.5 % trichloracetic acid for 30 min at room temperature
Alcian Blue stain: 12.5 % trichloracetic acid for 30 min at room temperature
Periodic acid Schiff (PAS) stain: 7.5 % acetic acid for 30 min at room temperature

Protein stain with Coomassie Blue

Prepare the solution:
Serva Blau G 0.2 g
(Serva-Heidelberg, Germany)
absolute ethanol 45.0 mL
distilled water 45.0 mL
glacial acetic acid 10.0 mL
Gel slabs are stained after prior fixation in this solution for 1 h at room temperature, then destained in 10 % acetic acid over several days.

Periodic acid Schiff stain

Gel slabs are taken from fixative, briefly rinsed in distilled water and immersed for 45 min at 4°C in 1 % periodic acid prepared in 3 % acetic acid; wash for 10 min (several changes) in distilled water and stain with Schiff reagent for 1 h at 4°C. Gels are destained in 10 % acetic acid.

Alcian Blue stain

Gels are taken from fixative and placed in 3 % acetic acid. Then, gel plates are immersed for 12 – 15 h in 0.5 % Alcian Blue 8GS (Chroma-Stuttgart, Germany) prepared in 3 % acetic acid. Gels are destained in 10 % acetic acid. All procedures are carried out at room temperature.

8.2 *Immunological Gel Diffusion*

Immunoelectrophoretic analysis and other techniques involving diffusion in gels can be performed in a variety of media. For practical purposes, 1 % agarose gel in sodium veronal-HCl buffer at pH 8.2 to 8.6 (0.025 – 0.05 mol/L) is useful.

Immunoelectrophoresis

1 % agarose in sodium veronal-HCl buffer is dissolved in a bath of boiling water and poured onto glass plates (situated horizontally in a template) until a layer of 2 mm thickness is built up. Templates in which anodic and cathodic junctions are cast in one mold are commercially available, thus enabling direct contact to be made with the respective electrode buffer containers. Holes for the probes (e.g. antigen mixture) and the lateral troughs for immune sera are cut out of the gelified medium using a commercially available cutting device. It is preferable to place the templates on a cooling block (connected to running tap water) during the electrophoretic run in order to avoid heating and drying of the gel matrix. The electrode buffer containers are filled with 0.05 mol/L sodium veronal-HCl buffer at pH 8.2 – 8.6.

Experimental procedure

Firstly, electrophoretic separation of the test sample is carried out with field strengths of about 6 V/cm until a color-labeled standard substance (e.g. bromophenol blue labeled albumin which is applied in a parallel track) has travelled the desired separation distance.
In the second step, precipitating immune sera are filled into the lateral troughs. Precipitation lines will develop from the side by overnight diffusion in a humid chamber.
Gel plates (on the glass slides) are then taken from the template, washed for at least 3 days in PBS (daily changes of the buffer solution), covered with filter paper, air-dried and stained in 0.1 % Amido black solution prepared in 1 mol/L acetic acid and 0.1 mol/L sodium acetate. Plates are destained in 2 % acetic acid to which 5 % glycerol has been added (to avoid cracking of the gel during air-drying). In the case of enzyme staining (e.g. HRP, GOD), gel plates are washed for 7 days, dried as above and tested for the respective enzyme by reaction with the appropriate substrate.

Double diffusion in gel

The same gel used for immunoelectrophoresis can be employed for Ouchterlony's technique of double diffusion in gel. Alternatively, gels can be prepared in PBS. Dissolved gel is poured onto glass slides or into Petri dishes to a depth of 2 mm. Holes (e.g. 3 mm in diameter) are cut in a defined arrangement into the cold gel and filled with antigen and antibody solutions, respectively. During overnight diffusion at room temperature, precipitation lines develop. The gels are then washed for 3 days in PBS, air-dried and stained; all procedures are analogous to those described for immunoelectrophoresis.

Radial immunodiffusion

The same gel matrix as described for immunoelectrophoresis or the technique of double diffusion in gel can be employed.

Technical procedure

An aliquot of 2 % agarose in distilled water (dissolved and kept at 56°C in a water bath) is mixed with an equal volume of antigen solution prepared in PBS and heated to 56°C. It should be noted that the amount of antigen must be established by trial and error in order to enable quantitation of antibody (or vice versa when antibody is incorporated into the gel). The mixture is poured onto strictly horizontal glass plates, which have previously been coated with a thin layer of 1.5 % agarose in distilled water and conditioned at 90°C for 1 h to enable firm attachment of the gel. The gel layer for radial immunodiffusion usually has a thickness of 2 mm.
Holes of 2.5 mm in diameter and 3 cm apart are cut into the jellified matrix and filled with 10 μl (exactly) of appropriate dilutions of immunoreactant; serial dilutions using a known standard are also used in order to calibrate the test system. Diffusion is allowed to proceed for 48 h in a humid chamber at room temperature. The plates are then washed, dried and stained as previously described (see immunoelectrophoresis).
The surface areas of the immunoprecipitates are calculated by measuring the ring diameters. The antibody concentration (antigen incorporated in the gel) varies linearly with the circle areas. The square of the radius is determined and this is directly proportional to the antibody concentration.
For antigen quantitation, antibody is incorporated into the gel and the preparation principles as described above are used (see Chapter 1.1.3 for references).

Electroimmunodiffusion

In Laurell's electroimmunodiffusion technique, specific antigen-antibody precipitates form peaks (rockets) which are stained by Amido black (Chapter 1.1.4). Apart from Amido black staining of such rockets, these can also be made visible by use of a second antibody con-

jugated with an enzyme. For this purpose, one can employ GOD labeled antibodies. Such a procedure, which needs only minor modification of the original method, makes the electroimmunodiffusion technique very sensitive and hence considerably lower concentrations of the samples can be quantitated; the threshold of detection is of the order of 10^{-7} g/mL.

Conventional method

Equal volumes of 2 % agarose in 0.05 mol/L veronal-HCl buffer at pH 8.2 to 8.6 (dissolved and maintained at 56°C) and prediluted immune serum (e.g. diluted 1:20 in veronal-HCl buffer; dilution established by trial and error) are mixed at 56°C in a water bath and poured onto glass plates (conditioned with 1.5 % agarose, see "Radial immunodiffusion"); the same template is used as that described for immunoelectrophoresis. Holes are cut (2.5 mm in diameter) in the cathodic area of the gel and filled with 10 µl (exactly) of the test sample; during filling, the electrophoretic voltage is reduced by a half. Then, plates are run for several hours (a potential of 5 V/cm migration distance is maintained), washed overnight in veronal-HCl buffer, air-dried under filter paper, and stained with Amido black. The height of the peaks is measured with millimetric paper. Results are plotted using a linear or semilogarithmic scale. Purified antigen (e.g. AFP) is used for the calibration curve; serial dilutions of 10 µg/mL to 0.5 µg/mL are made.

Enzyme-linked method

Preparation of the gel is the same as that for the conventional method. Only the primary immune serum incorporated into the gel is employed in much higher dilution (e.g.50 µg/mL gel). After electrophoresis of the samples (see above) rockets are not detectable by inspection or classical Amido black staining! For their visualization, a second antibody labeled with an enzyme must be employed. For this purpose, gel plates are first washed for 2 days in PBS and are then incubated with sheep anti-rabbit IgG antibodies conjugated with GOD (when the primary antibodies are from rabbit) for 12 h at room temperature at a concentration of about 0.05 mg/mL PBS. After washing in PBS for 7 days (2 changes of buffer per day), the plates are air-dried under filter paper and treated with a cytochemical stain to test for GOD activity (see Chapter 3.2).

8.3 Immunological Precipitation of Antigen and Antibody

Qualitative determination of the equivalence zone is necessary for the preparation of soluble peroxidase-antiperoxidase complexes (PAP). The principles of immunological precipitation of antigens by antibodies have been described by Heidelberger and Kendall [1]. In the following we give an example of how to proceed in the qualitative determination of the equivalence zone of a given rabbit anti-HRP immune serum, produced as described in Chapter 1.3.2.

Antigen solution

Solutions of HRP from 0.05 mg HRP/mL saline (0.15 mol/L NaCl) to "x" mg HRP/mL saline (up to 3 mg HRP/mL when anti-HRP immune sera of high titer are employed) are made up in increasing steps e.g. 0.05 mg/mL per tube.

First test series

0.2 mL of each diluted antigen step is added to numbered tubes (one tube for each dilution step). 0.2 mL of rabbit anti-HRP immune serum is then added to each tube and mixed. The tubes are left at 4°C over-

night and then centrifuged for 30 min at 5000 rpm/min. The supernatants are collected individually in numbered tubes of a *Second test series* (numbers correspond to numbers of first test series) and 0.15 mL of each supernatant transferred into numbered tubes of a *Third test series*.

Second test series

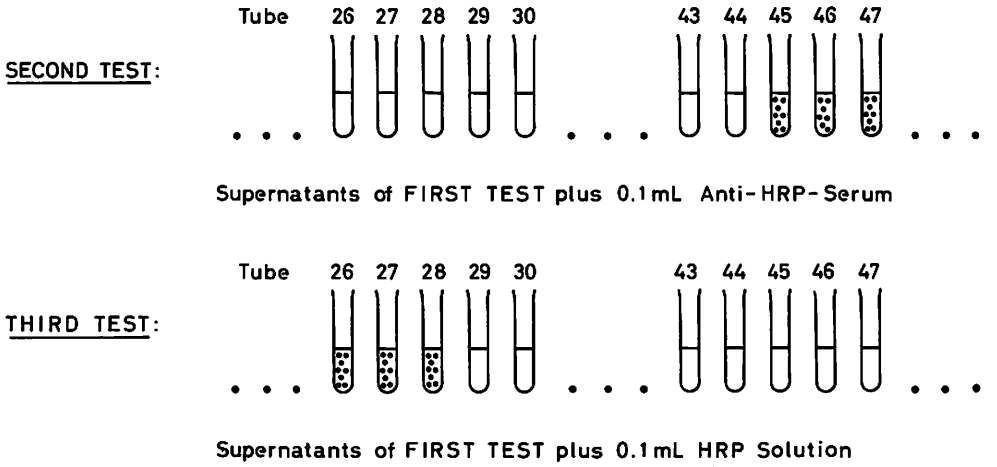
0.1 mL of anti-HRP immune serum is added to each tube. Precipitation will occur when the supernatants of the *First test series* still contain antigen. The presence of excess antigen in these supernatants is established by qualitative examination of formed precipitates after 1 h at room temperature.

Third test series

0.1 mL of 0.1 mg HRP/mL saline is added to each tube. Precipitation will occur when supernatants of the *First test series* still contain antibodies. The presence of excess antibody in these supernatants is established by qualitative examination of formed precipitates after 1 h at room temperature.

Evaluation of tests

Readings are performed with tubes containing:
(a) supernatants in the zone of excess antibody (observed in the *Third test series*);
(b) supernatants in the zone of excess antigen (observed in the *Second test series*);
(c) supernatants in the equivalence zone with no excess antibody or antigen (observed in both test series):



The concentration of HRP per mL *Antigen solution* (i.e. "Solutions of HRP from 0.05 mg/mL saline to "x" mg HRP/mL saline") is determined in that tube whose equivalence zone is nearest to the antigen excess zone. In the above experiment, this occurs with tube no. 44 and which corresponds to *2.2 mg HRP/mL*.

8.4 Chromatography and Gel Filtration

A variety of matrices and gel media can be obtained from commercial suppliers. The latter also supply precise laboratory guidelines for handling the gels in batch and column

procedures. This includes the use of matrices for coupling ligands (e.g. immunologically reactive, lectins etc.) to be employed in solid-phase affinity chromatography. Principles and typical experiments are given in Chapter 1.2.

Ion exchange chromatography

The choice of either an anion or a cation exchanger depends on the net charge of the molecules to be separated. Cellulose based or Sephadex based ion exchangers may be used. The porosity of an ion exchanger gel is determined by the type of matrix: its capacity depends largely on the ionic strength employed, pH value and porosity of the matrix. It should be noted that swelling of the ion exchanger is also influenced by ionic strength and pH. The capacity of an ion exchanger is usually given with the batch or can be determined experimentally; for DEAE Sephadex A-50, reversible binding of hemoglobin with 0.01 mol/L buffer at pH 8.0 is in the order of 5 g hemoglobin per g dry ion exchanger. Analytical and preparative column methods including equipment, equilibration of ion exchangers, preparation of the column bed, addition and binding of components to be separated, desorption and spectrophotometric detection are fully described in suppliers' manuals and will not be described here.

Gel filtration

A selection of virtually inert matrices for molecular size sieving of biomolecules is given in Table 1 – 2. The principles involved and an experimental run are described in Chapter 1.2.2. A detailed description of gel properties, the design of experiments for a variety of purposes and procedures for handling gels under laboratory conditions are provided in suppliers' manuals.

8.5 *Spectrophotometrical Methods*

Quantitative determination of proteins

The method of Lowry et al. [2] is described. Bovine serum albumin is taken as standard and used in increasing concentrations in order to establish a calibration curve.

Preparation of the protein standard

Exact amounts of crystalline bovine serum albumin (BSA) are weighed into individual tubes and dissolved in PBS to give a series of defined concentrations in the tubes, e.g. 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL and 1.0 mg/mL, respectively.

Preparation of solutions:

solution I	2 % Na_2CO_3 (water free) in 0.1 mol/L NaOH
solution II	1 % $CuSO_4 \cdot 5\,H_2O$ in distilled water
solution III	2 % K-Na-tartrate in distilled water
solution IV	mix solution II and solution III (ratio 1 : 1, vol./vol.); freshly prepared prior to use
solution V	mix 1 mL of solution IV with 50 mL of solution I; freshly prepared prior to use
solution VI	mix 5 mL of Folin-Ciocalteu phenol reagent with 4.7 mL of distilled water; freshly prepared prior to use

Assay

Wavelength = 748 nm; light path = 1.0 cm; final volume = 2.85 mL; blank = PBS; all measurements made at room temperature

0.1 mL of each of the serial dilutions of the protein standard and of the unknown test sample are pipetted into individual tubes; 0.1 mL PBS is used as blank. 2.5 mL of solution V is then added to each tube, mixed and allowed to stand for 12 – 15 min. 0.25 mL of phenol reagent (solution VI) is added, mixed rapidly and allowed to stand for 30 min. Finally, absorption measurements are carried out in the spectrophotometer.

The calibration curve is obtained by plotting standard protein concentrations on the abscissa and absorbance values of their respective color reactions on the ordinate of regular millimetric paper.

Quantitation of peroxidase activity

For the quantitative measurement of peroxidase activity in the spectrophotometer the following method is used.

Principle

$$H_2O_2 + DH_2 \xrightarrow{HRP} 2\,H_2O + D$$

(DH_2 = Leuco-dye; D = dye)

A large number of dyes exist for the detection of peroxidase activity. Here, we give a widely employed procedure [3].

Assay

Wavelength = 436 nm; light path = 1 cm; final volume = 3.11 mL; blank = phosphate buffer; $\epsilon_{436\,nm} = 6.39$ [$cm^2/\mu mole$]; temperature = 25°C;
Δ E/min (change in optical density in the spectrophotometer at 436 nm per unit time) is used to calculate peroxidase activity.

Pipette into cuvette:
3.00 mL of 0.1 mol/L phosphate buffer pH 7.0
+ 0.05 mL guaiacol (245 mg/100 mL distilled water)
+ 0.02 mL sample (ca. 0.002 mg of HRP/mL)
mix and start the reaction by adding
0.04 mL H_2O_2 solution (1.0 μL of 30 % H_2O_2/mL phosphate buffer)
mix and read change in optical density per min: when the extinction is between E = 0.2 and E = 0.25, then read change after 1, 2 and 3 min and calculate Δ E/min (mean value).

Calculation

$$\text{Volume activity} = \frac{3.11}{6.39 \times 1 \times 0.02} \cdot \Delta\,\text{E/min (U/mL sample)}$$

$$\text{Specific activity} = \frac{\text{volume activity}}{\text{concentration}}$$

Specific activity of HRP RZ 3.0 is approximately 250 U/mg lyophilized enzyme measured with guaiacol; U/mg protein, protein determination according to Lowry et al. [2].

Quantitation of glucose oxidase activity

For glucose oxidase (GOD) conjugation of antibodies, the quantitative measurements of GOD activity follow the principle described by Bergmeyer [3]

Principle

$$\text{I}\quad \text{D-glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$\text{II}\quad H_2O_2 + DH_2 \xrightarrow{\text{HRP}} 2\,H_2O + D$$

(DH_2 = Leuco-dye; D = dye)

Assay

Wavelength = 436 nm; light path = 1.0 cm; final volume = 3.0 mL; blank = phosphate buffer; temperature = 25°C; $\epsilon_{436\,nm} = 8.3$ [cm²/μmole];

Pipette into cuvette:

2.47 mL of o-dianisidine solution (0.066 mg o-dianisidine·HCl/mL 0.1 mol/L phosphate buffer pH 7.0); buffer previously saturated with O_2 for 10 min
+ 0.50 mL D-glucose solution (100 mg/mL buffer)
+ 0.01 mL of HRP solution (2 mg HRP RZ 3.0 per mL buffer)

mix and start the reaction by addition of

0.02 mL sample (ca. 0.002 mg GOD/mL)

mix and read change in optical density per min: changes after 1, 2, 3, 4 and 5 min are measured; calculate Δ E/min (mean value).

Calculation

$$\text{Volume activity} = \frac{3.00}{8.3 \times 1.0 \times 0.02} \cdot \Delta\,\text{E/min (U/mL sample)}$$

$$\text{Specific activity} = \frac{\text{volume activity}}{\text{concentration}}$$

Commercially available GOD from *Aspergillus niger* can possess a range of different specific activities. For labeling purposes, activities in the order of 210 U/mg are preferred.

Estimation of soluble PAP complexes

The production of soluble peroxidase-antiperoxidase antibody complexes (PAP) is described in Chapter 2.4; the HRP content and the molar ratio of HRP to anti-HRP in PAP can be determined using the method of Sternberger [4, 5]; samples are diluted 1 : 10 in PBS.

HRP content per mL

$A_{400\,nm} \times 0.413 \times 10\,mg$

Anti-HRP content per mL

$$\left[A_{280\ nm}\ \text{of PAP} - \frac{A_{400\ nm}\ \text{of PAP} \times A_{280\ nm}\ \text{of HRP}}{A_{400\ nm}\ \text{of HRP}} \right] \times 0.62 \times 10\ \text{mg}$$

Molar ratio of HRP to anti-HRP in PAP

$$\frac{\text{mg HRP} \times 156\,000}{\text{mg anti-HRP} \times 39\,800}$$

Enzymatic activity

Residual enzymatic activity of HRP in PAP is expressed by the formula [5]:

$$\frac{\text{(Enzymatic activity per mole HRP in PAP)} \times 100}{\text{(Enzymatic activity per mole HRP used for preparation of PAP)}}$$

Peroxidase activity is determined by the procedure described in "Quantitation of peroxidase activity".

8.6 *Histological Preparations*

Here we describe only a selection of procedures (published and unpublished) which we have found useful for the immunological and lectin stainings of organs. For detailed description of other technique the reader is referred to textbooks of histochemistry, pathology, and cell biology.

Preparation of formaldehyde and glutaraldehyde

Formaldehyde

Solutions can be prepared from paraformaldehyde when stock solutions of commercial origin (routinely employed in histopathology) prove unsuccessful: this is often due to presence of contaminants such as methanol. Paraformaldehyde dissolves readily under mild alkaline conditions in hot water [6]. For the preparation of a tissue fixative of e.g. 8 % formaldehyde in cacodylate buffer pH 7.2 the procedure is as follows:

Firstly, a stock solution of 16 % formaldehyde is prepared: 16 g of paraformaldehyde is weighed out, 80 mL distilled water is added and the mixture heated in a water bath to approximately 70°C (the vial is kept well stoppered). 1 – 2 mL of 1 mol/L NaOH is then added and the mixture shaken gently; the solution immediately becomes clear. The liquid is cooled and filtered (paper filter) and adjusted with distilled water to give 100 mL total volume.
Prior to use, this stock solution is diluted 1 : 1 (vol./vol.) with e.g. 0.2 mol/L cacodylate buffer pH 7.2 to give a final concentration of 8 % formaldehyde.

Glutaraldehyde

Glutaraldehyde for use as fixative for biological material or as bifunctional reagent for the preparation of enzyme-antibody conjugates should be of high purity. Besides the mon-

omer, commercial glutaraldehyde solutions often contain amounts of polymers, α, β-unsaturated aldehydes produced by aldol condensation, glutaric acid, and inorganic substances (cf. Beilstein's Handbuch der Organischen Chemie E III 1.3111) whose presence can cause unexpected reactions. For this reason, the degree of purity must be checked regularly prior to use. This is calculated from spectrophotometric absorptions; the purification index (P.I.) is expressed by P.I. = $E_{235\ nm} : E_{280\ nm}$ and should be better than 0.2 for the present purpose. The smaller the value of the P.I. the greater is the degree of purity. If necessary, glutaraldehyde can be purified by vacuum distillation [7, 8] using a Vigreux column. Some commercially available glutaraldehyde solutions have been compared and data given in Table 8 – 1 show the range of their degrees of purity.

Table 8-1: Degree of Purity (PI = $E_{235} : E_{280}$) of Different Commercial Glutaraldehydes.

Product	$E_{235\ nm}$	$E_{280\ nm}$	Purification index
Merck lot 9659091 25 % sol. EM grade	0.2	0.36	0.55
Serva lot 21050 25 % sol. purified	0.09	0.38	0.24
Sigma lot 99C-5029 25 % sol. grade I	0.1	0.32	0.31
Polysciences lot 01926 50 % sol. biol. grade	0.89	0.26	3.42
Polysciences lot 02291 70 % sol. EM grade	0.01	0.34	0.03

BSA encapsulation

Encapsulation of cell suspensions, cell organelles, bacteria, viruses etc. is often desirable in order to prepare "blocks" which can be handled like classical tissue blocks [9]. Such encapsulation procedures prove useful after immuno-staining of specimens.

Suspensions (cells, particles etc.) are fixed in aldehydes, washed and immuno-stained (if wanted) followed (possibly) by postfixation. The respective washing and incubation solutions are changed after light centrifugation.

Pellets are resuspended in 10 – 15 % BSA (bovine serum albumin) made e.g. in cacodylate buffer, transferred into a collodion bag (usually used for ultrafiltration and obtainable from Sartorius-Göttingen,

Germany) and centrifuged for 5 – 10 min at ca. 2000 rpm. Supernatant BSA is removed, the pellet is covered with a drop of 10 % glutaraldehyde solution, and the collodion bag is placed in a tube containing 10 % glutaraldehyde. Glutaraldehyde penetrates the bag and polymerizes cells containing BSA.
The collodion bag is removed and blocks are cut with a razor blade. These are washed, changing the buffer solution several times, and processed for light or electron microscopy in the usual way.

This procedure can also be applied to aldehyde-fixed cell suspensions, bacteria, viruses or tissue blocks for cryo-ultramicrotomy [9]. Such a step can lead to enhancement of the spreading quality of ultrathin cut frozen sections on the surface of the liquid in the knife trough.

Organ fixation and paraffin embedment

For a detailed description of all available tissue fixations in routine, dehydration schedules and embedments for classical histopathological purposes see references [10] and textbooks of histopathology. Two representative schedules which we employ in our immunohistological work are given.

Tissue blocks of ca. 2 – 3 mm are fixed in solutions of buffered formaldehyde, glutaraldehyde or combined mixtures of both at 0 °C. During fixation, solutions are changed at least twice; for appropriate fixation see Chapter 4.1. After being washed overnight at 4°C in buffer (several changes), blocks are dehydrated in solutions of ethanol of increasing concentration. Dehydration is performed at 0 – 4°C until 100 % ethanol is reached. The steps in 100 % ethanol depend on the tissue size. Benzene or chloroform are used as intermedium at room temperature prior to impregnation in hot paraffin. Special low-temperature melting paraffin appears not to be important. We use successfully conventional paraffin (melting point 56 – 57°C). A typical schedule is:

30 % v/v ethanol/water	30 min
50 % v/v ethanol/water	30 min
70 % v/v ethanol/water	30 min
90 % v/v ethanol/water	30 min
95 % v/v ethanol/water	30 min
100 % ethanol	4 × 60 min
chloroform	3 × 60 min
paraffin	120 min
paraffin	120 min or overnight

For numerous immunohistological studies, fixation in 99 % ethanol -1 % acetic acid at 0°C is preferred [11]. Large tissue blocks (slices of ca. 0.5 cm thickness) are fixed for 12 – 24 h while bioptic material needs less time, e.g. 3 hours. After fixation, blocks are transferred for dehydration to 100 % ethanol at 0°C. A typical schedule is:

100 % ethanol, 0°C	60 min
100 % ethanol, room temperature	3 × 60 min
chloroform	3 × 60 min
paraffin	120 min
paraffin	120 min or overnight

Lengths of time in ethanol and chloroform depend largely on tissue size.

Dehydration and embedment in Epon 812

Biological specimens for ultrastructural studies are embedded in one of the previously described resins (Chapter 4.3) for electron microscopy. Such embedment is also useful for studies of structural details with the light microscope. Dehydration and embedment are carried out after (a) double fixation of tissue by aldehydes and OsO_4 (immuno-staining may be performed in the preembedding period and is usually performed after aldehyde stabilization) or (b) immediately after aldehyde fixation (then, immuno-staining may be performed in the postembedding period on thin resin sections).

Standard method after double fixation (room temperature)

70 % v/v ethanol/water	20 min
95 % v/v ethanol/water	20 min
100 % ethanol	3 × 20 min
propylene oxide	2 × 30 min
propylene oxide/Epon mixture (ratio 1 : 1, Epon mixture see Table 4 – 3)	60 min
Epon mixture	120 min at 37°C or overnight at room temperature

embedment in capsules or flat molds with fresh Epon mixture at 60°C for 18 – 24 hours. For further information see reference [12].

Modification for postembedment immunohistology

Dehydration is preferably done at 0 – 4°C until 100 % ethanol is reached (see Chapters 4.2 and 4.3). A typical schedule is:

30 % v/v ethanol/water	30 min
50 % v/v ethanol/water	30 min
70 % v/v ethanol/water	30 min
90 % v/v ethanol/water	30 min
95 % v/v ethanol/water	30 min
100 % ethanol	6 × 20 min
propylene oxide	2 × 30 min
propylene oxide/Epon mixture (ratio 1 : 1, Epon mixture see Table 4 – 3)	60 min
Epon mixture	60 min at 35°C

embedment in fresh Epon mixture by transfer into capsules or flat molds;

polymerization in three steps:	35°C/24 h
	45°C/24 h
	60°C/24 h

Autoradiography for light microscopy

The stripping-film technique after Pelc [13] is a useful method for which commercial fine grain autoradiographic films (Kodak Stripping Plate AR. 10) are available.

Preparation of histological slides

Histological preparations are deparaffinized and immuno-stained as described in Chapter 5.1, but are not mounted under coverglass; semithin resin sections can also be used (Chapter 5.3).

Preparation of the pretreating solution:	
pure gelatin	2.5 g
distilled water	500 mL
dissolve at 60°C, then cool to room temperature and add	
potassium chromium (III) sulfate	0.25 g
the solution is filtered prior to use.	

Air-dried slides are placed in potassium chromium-gelatin solution for 30 – 60 sec and air-dried again.

Application of stripping film and exposure

Slides are taken into the dark room (18°C), illuminated only by a red safelight (Wratten series no. 1, 25 W bulb); distance of light source from film material should be at least 1 meter. Stripping film is removed from the refrigerator (4°C) about 1 h prior to use.

Stripping plate is taken from the packet and the area of the emulsion with its inert gelatin support is cut with a sharp razor blade into regular pieces. Their size must be larger than that of the histological specimen and must be large enough to overlap the slide. A strip is carefully removed from the plate by freeing a corner with a smooth, blunt scalpel. The film is slowly peeled from the glass, inverted and floated emulsion side downwards on distilled water containing 0.01 % potassium bromide.

Film strips are allowed to swell for at least 2 – 3 min. Individual strips of film are then removed with histological specimens facing upwards (towards the emulsion). Slides are air-dried at room temperature for several hours.

For exposure, specimens are placed in lighttight boxes and stored at 4°C in dry atmosphere. Test exposures are made at different time intervals in order to determine the optimal exposure time.

Development and fixation of autoradiographs

Prepare the developer solution:	
p-methylaminophenol sulfate	0.4 g
(Photo-Rex, Merck-Darmstadt, Germany)	
sodium sulfite anhydrous	18.0 g
distilled water	100 mL
dissolve on magnetic stirrer and add	
hydroquinone	1.6 g
distilled water	100 mL
sodium carbonate 1- hydrate	9.0 g
potassium bromide	1.0 g
Prepare the fixing solution:	
Kodak "Unifix" powder	26.0 g
distilled water	100 mL

Slides are placed (under red safelight) in slide dishes, which are filled with developer and developed for 5 min. Specimens are then rinsed for 10 – 20 sec in distilled water and fixed for 15 min; all solutions are at room temperature (18°C). After being fixed, the slides are washed under running tap water for 30 min. Finally, autoradiographs are air-dried in a dust-free atmosphere. Immunoperoxidase stained specimens are not further stained. After being thoroughly dried the samples are mounted under coverglass in Kaiser's glycerol-gelatin or in a

resinous medium (do not dehydrate in ethanol and do not clear in xylene since solvents do not penetrate emulsion and gelatin).

Staining of paraffin sections

Experimental details for immuno-staining paraffin sections are described in Chapter 5.1. Apart from specific immunological localization of cellular molecules by use of antibodies, counterstaining of sections with dyes and defined cytochemical reactions give further, important histological information. Some easily performed procedures are given here. For a review of general histological stains see reference [10].

Conventional hematoxylin-eosin stain

Paraffin sections are passed successively through xylene, 100 % ethanol and solutions containing decreasing amounts of ethanol until distilled water is reached. Staining is performed as follows:

Mayer's hemalum solution, filtered (Merck-Darmstadt, Germany)	9 min
running tap water	60 min
0.5 % eosin in distilled water	1 min
distilled water	2 × 1 min
70 % v/v ethanol/water	2 × 2 min
95 % v/v ethanol/water	2 × 2 min
100 % ethanol	2 × 2 min
xylene	2 × 5 min

mounting under coverglass in resinous medium, e.g. Eukitt (Kindler-Freiburg, Germany)

Hemalum counterstain of immunoperoxidase reacted sections

Immunoperoxidase sections (with or without postfixation in OsO_4) are passed through distilled water and stained by a modification of the previously described hematoxylin-eosin method:

Mayer's hemalum solution, filtered	2 – 5 min
running tap water	60 min
distilled water	1 min
70 % v/v ethanol/water	2 × 1 min
95 % v/v ethanol/water	2 × 1min
100 % ethanol	2 × 2 min
xylene	2 × 5 min

mounted under coverglass in resinous medium

Methyl green counterstain

Immunoperoxidase sections are passed through distilled water and stained:

0.1 % methyl green in distilled water (Merck-Darmstadt, Germany)	10 min
70 % v/v ethanol/water (for differentiation)	10 sec

95 % v/v ethanol/water	1 min
100 % ethanol	2 × 2 min
xylene	2 × 5 min

mounted under coverglass in resinous medium

Periodic acid Schiff (PAS) reaction

Sections, either immunoperoxidase stained or not, are passed through distilled water.

Prepare the solutions:
0.8 % periodic acid in distilled water,
disulfite wash solution (mixture of 20 mL 10 % sodium disulfite in distilled water, 20 mL 1 mol/L HCl in distilled water, 400 mL distilled water)

Staining procedure:

periodic acid solution	10 min
distilled water	2 × 5 min
Schiff reagent (Chroma-Stuttgart, Germany)	20 min
disulfite wash solution	2 × 6 min
distilled water	5 min
70 % v/v ethanol/water	2 × 1 min
95 % v/v ethanol/water	2 × 1 min
100 % ethanol	2 × 2 min
xylene	2 × 5 min

mounted under coverglass in resinous medium

Staining with Alcian Blue at pH 2.5

Immunoperoxidase reacted as well as routine histological sections are passed through distilled water and stained with Alcian Blue.

Prepare the solution:
0.1 % Alcian Blue 8GX/8GS (e.g. from Chroma-Stuttgart, Germany) in acetic acid at pH 2.5 (add glacial acetic acid to distilled water to give pH 2.5, which corresponds to approximately 3 % acetic acid in distilled water)

Staining procedure:

3 % acetic acid in distilled water	2 min
Alcian Blue solution	30 min
distilled water	2 rinses
70 % v/v ethanol/water	2 × 1 min
95 % v/v ethanol/water	2 × 1 min
100 % ethanol	2 × 2 min
xylene	2 × 5 min

mounted under coverglass in resinous medium

Carbohydrate staining by lectin-peroxidase conjugates

In a similar way to peroxidase labeled antibodies, lectins can be conjugated with peroxidase and employed for the histological localization of carbohydrate structures in tissues. Lectins possess a high affinity and a narrow range of specificity for definite sugar residues [14 – 17] and, thus, are useful tools for the characterization of carbohydrate moieties in cellular glycoproteins [18 – 23].

General staining procedure:
Paraffin sections are passed successively through xylene, 100 % ethanol and decreasing concentrations of ethanol and finally through PBS.
Inhibition of endogenous peroxidase activities by incubation in 1 % H_2O_2/PBS for 60 min at room temperature, followed by three successive washings in PBS of 5 min each.
Incubation in HRP labeled lectins (0.001 – 0.02 mg/mL PBS) for 18 – 24 h at 4°C, followed by three successive washings in PBS of 5 min each. Controls for specificity consist in incubation in HRP labeled lectins supplemented with either of the respective inhibitors.
Demonstration of HRP activity by incubation in 3,3'-diaminobenzidine and H_2O_2 [24] as described in Chapter 3.2. The cytochemical reaction product can be intensified by postfixation in 0.1 % OsO_4/PBS for 1 min; excess of OsO_4 is washed off with 70 % ethanol.
Slides are dehydrated in ascending series of ethanol, passed through xylene and mounted under coverglass in resinous medium.

Staining of semithin resin sections

Semithin sections from Epon embedded tissues are readily stained with Azur-Methylene blue [25].

Prepare the solutions:
1 % periodic acid in distilled water
1 % Azur blue in distilled water (Azur blue = Azur I or Azur B)
1 % Methylene blue in 1 % sodium tetraborate (Borax)

Proceed to staining:
Semithin sections are deposited on acetone cleaned slides and dried for 30 min at 90°C.
In the case of OsO_4 fixation, preparations are reacted with periodic acid for 5 min at room temperature, rinsed with distilled water and dried at 90°C. Sections without OsO_4 fixation are stained directly.
Solutions of Azur blue and Methylene blue are mixed 1 : 1 (vol./vol.); cold sections are covered with the staining solution and reacted at 90°C for 3 – 4 min. Slides are then transferred to the cold and rinsed with excess of distilled water.
Sections are differentiated in 70 % ethanol for ca. 10 sec, dehydrated in 100 % ethanol, passed through xylene and mounted under coverglass in resinous medium.

Counterstains for ultrathin Epon sections

Apart from specific immunological "staining" of defined cellular constituents by labelled antibodies, so-called nonspecific stainings with heavy metal salts, such as those of uranium and lead can be used [26]. These stains are very useful for checking the quality of specimen preservation in electron microscopic preparations. The use of solutions of lead salts at high pH for short periods of time is convenient for enhancing the contrast of immunoperoxidase reactions in cells.

Alkaline lead stain [27]

Prepare the solutions:
1 mol/L lead (II) nitrate in distilled water
1 mol/L tri-sodium citrate dihydrate in distilled water
1 mol/L sodium hydroxide in distilled water
(solutions are filtered prior to use)

Staining solution:
16 mL of freshly distilled water is mixed with 3 mL of sodium citrate solution. 2 mL of lead nitrate is then added dropwise with continuous stirring. Any white precipitates dissolve readily upon addition of 4 mL of sodium hydroxide solution. The final staining solution must be completely clear.

Staining procedure:
For each grid to be stained, one drop of the staining solution is deposited on a clean covered wax plate (e.g. placed in a Petri dish containing sodium hydroxide to remove carbon dioxide; avoid contamination with CO_2!). Sections (immuno-stained) are reacted for no longer than 30 sec to 1 min. For conventional ultrastructural studies, sections previously stained with uranium salts are usually stained for 5 min.
Grids are transferred by tweezers to droplets of 0.005 mol/L sodium hydroxide in distilled water (2 passages), rinsed with distilled water and finally dried by placing the grids on filter paper.

Uranium salt staining

Prepare the solutions:
5 % uranyl acetate in distilled water (filtered 12 h after preparation)
0.1 mol/L hydrochloric acid in distilled water
Michaelis buffer (modified: 1.17 g sodium acetate anhydrous
2.94 g diethylbarbituric acid sodium salt
made up with distilled water to 100 mL)

Staining solution:
13 mL of uranyl acetate is mixed with 7 mL of hydrochloric acid and 5 mL of Michaelis buffer is then added. Tubes containing the solution are tightly stoppered and stored at room temperature in the dark; centrifuge prior to use.

Staining procedure:
A watch glass is filled with staining solution and the specimen grid (sections downwards) is floated on the liquid surface for 20 min.
At the end of the staining period, the grid is removed (do not dry) and quickly rinsed under a jet of water from a wash bottle (distilled water). The grid is then dried by placing it on filter paper (sections uppermost).

References

1. M. Heidelberger and F.E. Kendall, "A quantitative theory of the precipitin reaction. III. The reaction between crystalline egg albumin and its homologous antibody", *J. Exp. Med.* **62** (1935) 697.
2. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, "Protein measurement with the Folin phenol reagent", *J. Biol. Chem.* **193** (1951) 265.
3. H.U. Bergmeyer: *Methods of Enzymatic Analysis.* Verlag Chemie, Weinheim-New York 1974.
4. L.A. Sternberger: *Immunocytochemistry*, J. Wiley & Sons, New York 1979.
5. L.A. Sternberger, P.H. Hardy, J.J. Cuculis and H.G. Meyer, "The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes", *J. Histochem. Cytochem.* **18** (1970) 315.
6. A. Reychler, "Beiträge zur Kenntnis der Eigenschaften des Formaldehyds", *Chem. Zentralblatt* **79** (1908) 714.
7. H.D. Fahimi and P. Drochmans, "Essais de standardization de la fixation au glutaraldéhyde. I. Purification et détermination de la concentration du glutaraldéhyde", *J. Microscopie* **4** (1965) 725.
8. P.J. Anderson, "Purification and quantitation of glutaraldehyde and its effects on several enzyme activities in skeletal muscle", *J. Histochem. Cytochem.* **15** (1967) 652.
9. W.D. Kuhlmann and A. Viron, "Cross-linked albumin as supporting matrix in ultrathin cryomicrotomy", *J. Ultrastruct. Res.* **41** (1972) 385.
10. B. Romeis: *Mikroskopische Technik.* R. Oldenbourg, München-Wien 1968.
11. W.D. Kuhlmann, "Alpha-fetoprotein: cellular origin of a biological marker in rat liver under various experimental conditions", *Virchows Arch. (Pathol. Anat.)* **393** (1981) 9.

12. A.M. Glauert: "Fixation, dehydration and embedding of biological specimens". In: A.M. Glauert (ed.): *Practical Methods in Electron Microscopy*. North-Holland Publ. Co., Amsterdam-Oxford, American Elsevier Publ. Col., New York 1975.
13. S.R. Pelc, "Autoradiograph technique", *Nature (Lond.)* **160** (1947) 749.
14. M.M. Burger and A.R. Goldberg, "Identification of a tumor-specific determinant on neoplastic cell surfaces", *Proc. Natl. Acad. Sci (USA)* **57** (1967) 359.
15. H. Lis and N. Sharon, "The biochemistry of plant lectins (phytohemagglutinins)", *Ann. Rev. Biochem.* **42** (1973) 541.
16. D.L. Simpson, D.R. Thorne and H.H. Loh, "Lectins: endogenous carbohydrate-binding proteins from vertebrate tissues: functional role in recognition processes?", *Life Sci.* **22** (1978) 727.
17. S.H. Barondes, "Lectins: their multiple endogenous cellular functions", *Ann. Rev. Biochem.* **50** (1981) 207.
18. G.L. Nicolson and S.J. Singer, "Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy: application to saccharides bound to cell membranes", *Proc. Natl. Acad. Sci. (USA)* **68** (1971) 942.
19. M.E. Etzler and M.L. Brandstrator, "Differential localization of cell surface and secretory components in rat intestinal epithelium by use of lectins", *J. Cell Biol.* **62** (1974) 329.
20. P.J. Stoward, S.S. Spicer and R.L. Miller, "Histochemical reactivity of peanut lectin-horseradish peroxidase conjugate", *J. Histochem. Cytochem.* **28** (1980) 979.
21. A. Sato and S.S. Spicer, "Ultrastructural visualization of galactose in the glycoprotein of gastric surface cells with a peanut lectin conjugate", *Histochem. J.* **14** (1982) 125.
22. C.R. Boland, C.K. Montgomery and Y.S. Kim, "Alterations in human colonic mucin occuring with cellular differentiation and malignant transformation", *Prog. Natl. Acad. Sci. (USA)* **79** (1982) 2051.
23. W.D. Kuhlmann, P. Peschke and K. Wurster, "Lectin-peroxidase conjugates in histopathology of gastrointestinal mucosa", *Virchows Arch. (Pathol. Anat.)* **398** (1983) 319.
24. R.C. Graham and M.J. Karnovsky, "The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique", *J. Histochem. Cytochem.* **14** (1966) 291.
25. K.C. Richardson, L. Jarett and E.H. Finke, "Embedding in epoxy resins for ultrathin sectioning in electron microscopy", *Stain Technol.* **35** (1960) 313.
26. P.R. Lewis and D.P. Knight: "Staining methods of sectioned material". In: A.M. Glauert (ed.): *Practical Methods in Electron Microscopy*. North-Holland Publ. Co., Amsterdam-New York-Oxford 1977.
27. E.S. Reynolds, "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy", *J. Cell Biol.* **17** (1963) 208.

Index

A

Absorption, antibodies 36
Adenovirus 122
Adsorbents *see* affinity chromatography
Adsorption, non-specific 30, 86, 133
Affinity chromatography 28 ff
–, alpha-fetoprotein 30
–, antibodies 37
–, antibody-peroxidase conjugates 50 ff
–, Concanavalin A 50 f
–, desorption 29 f
–, specific elution 30
Agarose derivatization 29 f
AgX *see* peroxidase-antiperoxidase complexes, preparation
Alcian blue
–, electrophoresis 139
–, glycoconjugates *see* glycoconjugates, acid
–, histology 152
Alpha-fetoprotein
–, electrophoresis 8
–, gel diffusion techniques 10 ff
–, isolation 30
–, localization 68, 97 ff, 113 ff
–, quantitation 12 f
Amido black stain 140
ε-amino groups
–, amidination 82
–, conjugation 47 f
–, fixation 73
Antibodies
–, analysis 34 ff
–, cellular basis 31 ff
–, cross-reactivity 34 ff, 133
–, Fab fragment 33, 38
–, hybrid 62
–, IgG class 33, 37
–, immune response 34
–, isolation 36 ff
–, monoclonal 32 f
–, natural 34
–, polyclonal 32 ff
–, preparation 31 ff
–, specificity 34 ff
–, structure 32 ff
Antibody conjugation *see* conjugation
Antigen detection
–, monolayer 101 ff
–, paraffin sections 85 f, 87 ff
–, resin sections 96 ff, 116 ff
–, ultrathin frozen sections 121 ff
Antigen determinants 32 ff, 73 ff
–, denaturation *see* denaturation
Antigen protection 79 ff, 98, 100
–, EAI 82 f
Antigen structure
–, conformational change 74
–, effect of fixation 74
–, polypeptide chain 73 f
Artefacts *see* specificity
Avidin-biotin 45
Autoradiography 65, 149 ff
Azur-Methylene blue stain 153

B

Background reactions *see* specificity
Bifunctional reagents 46
Biological specimens
–, cell suspensions 71, 108 ff
–, cryostat sections 71, 108 ff
–, cryo-ultramicrotomy 121 ff
–, dehydration 75 f, 148 f
–, embedment in paraffin 76 f, 148
–, embedment in resin 77 ff, 149

– , encapsulation in BSA 147 f
– , fixation formaldehyde 72 ff, 131 f, 148
– , fixation glutaraldehyde 73 ff, 131 f, 148
– , fixation organic solvents 90, 101, 148
– , general preparations 71 ff
– , lectin staining 152 f
– , methods immuno-staining *see* immuno-histology
– , monolayer 71, 101 ff, 122 ff
– , solid organs 71 ff, 85 f, 90, 97 f, 113
Bioselective affinity *see* affinity chromatography
Booster injection *see* immunization

C
Chaotropic ions 29 f
Chessboard titration 63 f
Chromatofocusing
– , amphoteric buffer 24 f
– , gastric mucosa glycoconjugates 25
– , principle 23 ff
Chromatography 142 f
Colloidal gold 44
Concanavalin A
– , affinity chromatography 50 f
– , cellular binding 113
Conformational change
– , dehydration 76
– , embedment 77, 79
– , fixation 74
Conjugates
– , characterization 51 ff
– , enzyme activity 54
– , immunoelectrophoresis 51, 53 f
– , immunological activity 54
– , immuno-staining *see* immunohistology
– , molecular weight 54
– , one-stage 53 f
– , purification 50 ff
– , SDS polyacrylamide electrophoresis 54
– , storage 49, 55
– , two-stage 53 f
Conjugation
– , antibody-enzyme 47 ff
– , antibody-ferritin 47
– , antibody-glucose oxidase 49
– , antibody-peroxidase 49
– , glutaraldehyde 47 ff
– , one-stage reactions 46 f
– , reagents 46 ff
– , two-stage reactions 46 ff
Control
– , antibodies 5 ff
– , antigens 5 ff
Control reactions 66 f, 113, 117, 131 ff
Coomassie blue stain 139
Counterstain
– , electron microscopy 67 ff, 153 ff
– , light microscopy 67 f, 151 ff
Coupling reagents 46 ff
Covalent linkage
– , coupling reagents 46 ff
– , cyanogen bromide 29 f
Cross-linkage
– , fixation 73 f
– , reagents 46 ff
– , resin curing 78 f
Cryomicrotomy 71, 108 ff
– , flat embedment 113 ff
– , section manipulation 113
Cryo-protection
– , DMSO 113, 122
– , sucrose 123
Cryo-ultramicrotomy
– , inert support 123
– , method 121 ff
Cyanogen bromide 29 f
Cytochemistry
– , diaminobenzidine 64 f
– , glucose oxidase 65 f
– , peroxidase 63 ff

D
DAB *see* diaminobenzidine
Dehydration
– , agents 76
– , organs *see* biological specimens
Denaturation
– , dehydration 76
– , embedment 77 ff, 100 f
– , fixation 72 ff
Detergents
– , affinity chromatography 30
– , preembedment immuno-staining 109 ff
Diagnostic *see* immunohistology, application
Diaminobenzidine 64 f, 117 f, 120, 122 f, 135
Diffusion artefacts *see* specificity
Double diffusion in gel 10 f, 140
Double labeling 65

E
EAI *see* ethyl acetimidate
Ectopic staining 133
Electroimmunodiffusion 12 f, 140 f
– , alpha-fetoprotein 13
– , enzyme staining 13, 141
Electrophoresis 6 ff
– , alpha-fetoprotein 7
– , analytical polyacrylamide 6 ff, 20, 28, 137 ff
– , disc 7
– , electrophoretic run 138 f

–, flat gel slab 138
–, gel bridges 26
–, glycoconjugates 8 f, 20, 27 f
–, isoelectric focusing 8
–, pore gradient 7
–, preparative polyacrylamide 15, 26 ff, 137 ff
–, sample preparation 138
–, SDS 7
–, separation gel 6 ff, 26, 138
–, stacking gel 26, 138
–, staining of gel 8, 139
–, supporting media 6 ff
–, tracking dye 138, 140
Embedment *see* biological specimens
Encapsulation, BSA 123, 147 f
Envelopment, BSA 123, 147 f
Enzyme conjugation 47 ff
Enzyme digestion 74, 79 f, 109
Enzymes *see* marker molecules
Enzyme substrates
–, glucose oxidase 65 f
–, peroxidase 65
Epon 78 ff, 96 ff, 117 ff, 149
Epon sections, antigens 97
Epoxy resin
–, exothermic profiles 79 f
–, manufacture 77 f
–, polymerization 78 f
Equivalence zone *see* AgX
Ethyl acetimidate 82, 97, 100
Evaluation
–, antibodies 5 ff
–, antigens 5 ff, 16
–, tissue sampling 107 f

F
False negative staining 66, 131 ff
False positive staining 66, 131 ff
Ferritin 44
Ferritin conjugation 47
Fixation
–, *in vitro* studies 74
–, organs *see* biological specimens
–, schedules *see* immunohistology, application
–, Schiff bases 73
Fixatives, organic or inorganic 72 ff
Formaldehyde fixation 72 ff, 146
–, classical 85
Formaldehyde preparation 146
Freeze-thaw cycle 108, 113
Freund's adjuvant 36

G
Gastrin 85 ff
Gastrointestinal antigens 88 ff
Gastrointestinal markers 88 ff
Gastrointestinal mucosa
–, differentiation 91 ff
–, glycoconjugates *see* glycoconjugates
–, histogenesis 91 f
–, marker molecules 88 ff
–, metaplasia 90 ff
–, mucus cells 90 ff
Gel filtration
–, applications 21 f
–, conjugates 50
–, fractionation range 21
–, glycoconjugates 22 ff
–, media 21
–, principle 19 ff, 142 f
Glucose oxidase
–, activity 145
–, Aspergillus niger 45
–, chromogens 65 f, 145
–, conjugation 49
Glutaraldehyde
–, commercial products 147
–, conjugation 47 ff
–, fixation 73 ff, 146 f
–, purity 48, 147
–, reaction with proteins 48
–, Schiff bases 73
Glycoconjugates
–, acid 8 f, 15, 89
–, blood group antigens 17, 88, 90
–, characterization 15 ff, 89 ff, 95 f
–, isolation from gastrointestinal mucosa 15 ff, 89 f
–, localization 90 ff
–, neutral 15 ff, 89 ff
Glycoproteins *see* glycoconjugates

H
Heavy metals
–, counterstains 153 f
–, immuno-labeling 44
Hemalum stain 151
Hematoxylin-eosin stain 151
Hemocyanin 44
Histology
–, antigen staining *see* immunohistology
–, conventional staining 151 ff
Histopathology *see also* immunohistology
–, gastric markers 88
Historical data 1 f
Horseradish peroxidase *see* peroxidase
HRP *see* horseradish peroxidase
Hyperimmunization *see* immunization

I
Immune response *see* antibodies
Immune sera

– , cross-reacting antibodies 34 f
– , preparation 31 ff
– , specificity 32 ff
– , unwanted antibodies 34
Immune system 31
Immunization 32 ff
– , rabbit 36
– , sheep 36
Immunoadsorption *see* affinity chromatography
Immuno-assays *see* quantitation
Immunodiffusion
– , gel techniques 6, 10 ff, 139 ff
– , Laurell *see* electroimmunodiffusion
– , radial 11 ff, 140
Immuno-electron microscopy *see* immunohistology, application
Immunoelectrophoresis 9 ff, 139 f
– , alpha-fetoprotein 10, 53
– , immune sera 10, 35
Immuno-enzyme techniques *see* immunohistology
Immunofluorescence 43
Immunoglobulins *see* antibodies
Immunohistology
– , alkaline phosphatase 109 f
– , alpha-fetoprotein 97 ff, 113 ff
– , antigens 85 ff, 107 ff
– , application of electron microscope 69, 107 ff
– , application of light microscope 68, 85 ff
– , avidin-biotin 62
– , bridge techniques 62
– , conjugates 61 ff, 85 ff, 108 ff
– , control reactions 66 f, 113, 117, 131 ff
– , diagnostic 85 ff, 107 ff
– , direct method 61, 108 ff
– , dispersive 125
– , enzyme substrates 63 ff, 111 ff, 117 f, 122
– , freeze-etching 125
– , gastrin 85 ff
– , glucose oxidase 109
– , glycoconjugates 90 ff
– , hybrid antibody 62
– , indirect methods 61 ff, 85 f, 90, 117
– , method cryo-section staining 113 ff, 121 ff
– , method monolayer staining 101 ff
– , method paraffin section staining 85 ff
– , method postembedding staining 85 ff, 116 ff
– , method preembedding staining 113 ff
– , method resin section staining 97 f, 117 f
– , miscellaneous techniques 125
– , PAP technique 62, 86
– , peroxidase 63 ff
– , protein A 61 f
– , scanning electron microscopy 125
– , specificity *see* specificity
– , T-antigen 101 ff
Immunolocalization *see* immunohistology
Immunological precipitation, methods 9 ff, 55 ff, 139 ff
Immunological reagents, analysis 5 ff
Immunopathology 85 ff
Immunoperoxidase staining 61 ff
Imposil 44
Incubation methods *see* immunohistology
Ion exchange chromatography
– , CM 17
– , DEAE 17 ff, 143
– , FPLC 19
– , glycoconjugates 19 f
– , HPLC 19
Isoeletric point 12 f, 23 f

L
Labeling
– , antibody *see* conjugation
– , biotin-avidin 44, 62
– , ferritin 44
– , gold 44, 101, 121
– , lectin 44, 125 f
– , protein A 44, 61 f
– , tissues *see* immunohistology
Latex particles 44
Lead stain 153
Lectin affinity chromatography 50 f
Lectin histology 152 f
Lectin-peroxidase conjugates 152 f
Ligand chromatography *see* affinity chromatography
Ligand immobilization *see* affinity chromatography
Ligand labeling 43 ff
Ligand lectin 152 ff
Localization
– , of antigens 61 ff, 85 ff, 107 ff
– , of ligands 61 ff
Lowicryl 77, 79, 81, 96, 121
Low-temperature, embedment 79, 121
Lymphoid cells
– , immune response 31
– , specific antibodies 31 f
– , ultrastructure 31 f

M
Marker enzymes 45
Marker molecules 43 ff
– , conjugation 46 ff
– , directly visible 44
– , electron microscopy 43 ff
– , enzymes 44 f
– , fluorescein 43
– , indirectly visible 44
– , light microscopy 43 ff
Masking of antigens 74, 79

Metaplasia
–, goblet cells 90 ff
–, intestinal 91 f
Methacrylate 77
Methylgreen counterstain 151
Microphotography
–, electron microscope 67
–, light microscope 67
Microscopy
–, electron microscope 67 ff
–, light microscope 67 ff
Modification of antigens 73 ff
–, reversal 79 ff
Mucins 89 ff
Mucus cells
–, histochemistry 89
–, immunohistology 90 ff
–, NGC substances 89 ff

N
Neutral glycoconjugates *see* glycoconjugates, neutral
NGC substances *see* neutral glycoconjugates

O
Organ *see* biological specimens
Organ extracts, isolation of proteins 14 ff

P
PAP *see* peroxidase-antiperoxidase complexes
Paraffin embedment 76 f, 148
–, routine histology 85
Paraformaldehyde *see* formaldehyde
PAS *see* periodic acid Schiff
Penetration studies
–, cell membranes 108 f
–, conjugates 108 ff
–, enzyme substrate 111 ff
–, marker molecules 108 ff
–, saponin 109 ff
–, tissue preparations 108 ff
–, Tween 109 ff
Periodic acid Schiff (PAS)
–, electrophoresis 139
–, histology 152
–, positive glycoconjugates 15, 19 f, 25 ff, 89 ff
Peroxidase
–, activation 49
–, activity 144 ff
–, chromogens 63 ff, 144
–, conjugation *see* conjugation
–, endogenous 133
–, enhancement of reactivity 65
–, horseradish 45
–, inhibition 85, 133
–, periodate oxidation 48
Peroxidase-antiperoxidase complexes
–, characterization 56, 144, 145 f
–, preparation 55 f, 141 f
–, storage 56
Peroxidase staining *see* immunohistology
pH gradient 23 ff
Pitfalls *see* specificity
Polyacrylamide gel *see* electrophoresis
Polyconjugates 46 f
Polymerization
–, conjugation 46, 48
–, exotherm profiles 79 f
–, fixation 72 ff, 147 f
–, resins 77 ff, 98, 149
Polyvinylpyrrolidones 81 ff, 97, 100
Postembedding localization
–, electron microscopy 116 ff
–, light microscopy 85 ff
Postfixation
–, aldehyde 120
–, osmium 113, 118, 120
Practical aspects 137 ff
Preembedding localization
–, electron microscopy 108 f
–, light microscopy 101 ff
Preembedment staining 113 ff
Pretreating media
–, immunohistology 85 f
–, resin removal 98 ff, 117 ff
Protease treatment 74, 79 f
Protein, net charge 17 f, 24
Protein A 45
Protein-protein coupling 46 ff
Protein stabilization
–, EAI 82 f
–, fixation *see* biological specimens
–, PVP 81 f
Purification of antigens 13 ff
Purity control
–, antibodies 5 ff
–, antigens 5 ff
PVP *see* polyvinylpyrrolidones

Q
Quantitation
–, antigen-antibody 11 f
–, conjugates 54
–, equivalence zone 141 f
–, gel diffusion techniques 139 ff
–, glucose oxidase activity 145
–, immune response 34
–, PAP complexes 145 f
–, peroxidase activity 144 f
–, proteins 143 f