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Organization of the Extracellular Matrix

A Polarization Microscopic Approach

László Módis

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Organization of the Extracellular Matrix: a Polarization Microscopic Approach

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PREFACE

Growing appreciation of the role played by connective tissues in various normal and pathological processes has led to the rapid development of research in this field. The extracellular matrix dominates the connective tissues, and is responsible for the basic functions of these tissues.

Furthermore, it is known that the extracellular matrix is also present in all tissues, and that it influences the differentiation, morphology, and functions of the cells. In light of this fact, matrix research has been attracting the intense interests of cell biologists, too.

Owing to the efforts of biochemists, much progress has been made in the exploration of the molecular anatomy of the matrix constituents. The contribution of morphologists is more modest due to the limitations of their methodology. Their role in contemporary matrix research may be compared to that played by Sancho Panza in Cervantes' classic tale, but we know that his thoughtful and dedicated service was invaluable to his master, and he proved to be a faithful and, at the same time, a helpful companion.

This volume is devoted to a peculiar morphological approach to matrix research. Polarization microscopy had been the leading technique of utrastructure research in the pre-electron microscopic era until electron microscopy provided the means of direct visualization of the ultrastructure, and the indirect techniques like polarization microscopy became more and more neglected.

The potentialities of polarization microscopy, however, were greatly enhanced when it became possible to combine them with relevant histochemical methods. These histochemical reactions, today called topo-optical reactions after the custom of G. Romhányi, a pioneer of this field, can selectively increase the optical anisotropy of a tissue constituent if it is spatially oriented. This modern polarization microscopy proved to be a powerful tool, especially in the exploration of oriented submicroscopic structures of different glycoconjugates like glycosaminoglycans in the matrix of different embryonic and differentiated animal tissues.

Despite the fact that these methods are being used more and more, we cannot call them well known. Therefore, when I was asked to write a volume, I decided that this contribution would be an attempt to display the state of the art of this research technique. Its advantages and limitations will be illustrated predominantly by the results achieved in our laboratory. Our work shows that the old assumption — believed even today by many researchers — that the matrix is an amorphous system, is no longer tenable.

It is hoped that the merits of this volume will overshadow its weaknesses. The author welcomes any constructive criticism and comments. *"Artes discuntur peccando."*

THE AUTHOR

László Módis, M.D., Ph.D., is Associate Professor of Anatomy, Histology, and Embryology at the University School of Medicine, Debrecen, Hungary.

Dr. Módis obtained his M.D. degree *(summa cum laude)* in 1963 and his Ph.D. in 1975, and served as an Assistant Professor from 1965 at the same University. He became Associate Professor in 1980. As a post-doctoral fellow, he spent 2 years in the Department of Histology and General Embryology, University of Fribourg, Switzerland (1969—1971) thanks to a grant from Swiss National Foundation for Scientific Research. More recently, as a visiting scientist, he spent 1 year (1989—1990) in the Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, supported by a NIH grant.

He is currently the elected Secretary of the Histochemical Section of the Hungarian Society of Anatomists, Histologists, and Embryologists, and member of the boards of the Hungarian Society of Anatomists, Histologists, and Embryologists, the Hungarian Connective Research Club, and the Osteoarthrological Society of Hungarian Orthopaedic Surgeons. He was the managing chairman of two international scientific meetings held in Debrecen (12th Symposium of the European Society of Osteoarthrology: Biology of the Cartilage, 1983; International Symposium on Histochemistry, 1985).

Among other awards, he has received the Lenhossék Medal from the Hungarian Society of Anatomists, Histologists, and Embryologists, and the title "Professor of the Year, 1981" from the medical students of his University.

Dr. Módis has published 90 papers and 1 book. He has presented over 30 invited lectures at International Meetings and over 20 invited seminars at different foreign Universities. His current major research interests include the differentiation of the structure of the extracellular matrix and cell-matrix interactions, predominantly in cartilage tissue.

TABLE OF CONTENTS

Chapter 1
A Short Overview of Major Constituents of the Extracellular Matrix 1

Chapter 2
Physical Backgrounds of Polarization Microscopy 9

Chapter 3
Topo-Optical Reactions Used in Polarization Microscopic Ultrastructure Research 31

Chapter 4
Structure of the Hyaline Cartilage Matrix 75

Chapter 5
The Organic Bone Matrix 99

Chapter 6
Structure of the Corneal Stroma 123

Chapter 7
Structure of Kidney Basement Membranes 137

Chapter 8
Experimental Reconstruction of the Oriented Glycosaminoglycan Structure of the Extracellular Matrix 157

Chapter 9
Factors Involved in Formation and Maintenance of Oriented Microstructure of Matrix Constituents 177

Chapter 10
Polarization Microscopy of Amyloid 207

Chapter 11
Correlation Between Polarization and Electron Microscopic Findings in Ultrastructure Research of Proteoglycans 229

Chapter 12
Methodological Appendix 265

Index 283

Dedicated to GEORGE ROMHÁNYI,
Emeritus Professor of Pathology, Pécs, Hungary,
one of the pioneers of polarization microscopy

Chapter 1

A SHORT OVERVIEW OF MAJOR CONSTITUENTS OF THE EXTRACELLULAR MATRIX

TABLE OF CONTENTS

I. Introduction 2

II. Major Constituents of the Matrix 2
- A. Collagen 2
- B. Elastin 4
- C. Proteoglycans 4
- D. Noncollagenous Proteins 4

III. Receptors Involved in Cell-Matrix Interactions 5

References 6

I. INTRODUCTION

The extracellular matrix (ECM) constitutes a significant part of the multicellular animal organism. Except blood, there are no tissues without an ECM. In connective tissue the matrix is abundant, while its bulk is much less in other tissues where sometimes special techniques are needed for its detection. The matrix molecules are produced and degraded by cells.

After deposition the ECM, or some of its constituents, influence a number of functions of contacting cells. The cell-matrix interactions are generally mediated by specific receptors in the cell membrane. The ECM can control the metabolism, growth, differentiation, migration, and adhesion of cells, the cytoskeletal organization and cell shape, and the organization of the tissues[1-5] (Figure 1). There are experimental data suggesting that ECM exerts an influence on gene expression via transmembrane proteins and cytoskeletal compartments which are associated with polyribosomes as well as the nuclear matrix.[6] According to this theory, the cytoskeletal association with polyribosomes may affect the rate of protein sythesis, while the matrix-cell membrane-cytoskeleton-nuclear matrix interaction could affect mRNA processing and rates of transcription.

Thus, the ECM is not a simple and passive structural scaffold. Multicellular animals can function only because single cells learned to synthesize, and maintain macromolecular connections between them, and they have become dependent upon the matrix they made.[7] This has been realized in the past decade due to the large amounts of new data accumulated about the biosynthesis, molecular anatomy, functions, and interactions of the ECM constituents. It is now, therefore, generally accepted that the interest of cell biology extends beyond the cell membrane.

There are different major trends in the contemporary ECM research. One of them is — the most classical — the biochemical approach with the aim of a detailed description of the matrix constituents and their interactions. The second and tremendously increasing field is the molecular biology of the ECM.[8] The third trend — which is rapidly developing — is the study of the role of the ECM in diseases.[9-11] A fourth direction is to get new data about the ECM structure in different normal and pathological tissues. In this respect, immunohistochemistry has contributed much to our present knowledge.[12] Other morphological techniques like electron microscopy and polarization microscopy, however, are also indispensable tools of ECM research. As this volume is dedicated to a morphological approach of the ECM organization, it is beyond the scope of the present work to give a detailed description of the ECM constituents. This chapter shortly summarizes some basic informations about the major macromolecular components of the ECM, other details are found in the references given as well as in the relevant chapters of this volume.

II. MAJOR CONSTITUENTS OF THE MATRIX

A. COLLAGEN

Collagen is the most ubiquitous protein in animal kingdom. It is a heterogenous family of proteins, but the members share common features both in primary structure and conformation, e.g., the repeating Gly-X-Y triplet in the polypeptide chain, and the presence of hydroxyproline and hydroxylysine.

Today, 14 collagens are distinguished. They differ genetically, chemically, and immunologically.[13-16] There are 5 of the collagen types which are known to be involved in the formation of crossbanded fibrils (types I, II, III, V, XI), but other collagens (e.g., type IX) may also be associated with the fibrillar surface. The collagen fibrils and fibers represent systems where the degree of the orientation of the macromolecular constituents is the highest in the ECM. Some collagen types are tissue-specific: types II, IX, X and XI are round in cartilage (and also in vitreous body and notochord) (see Chapter 4). Collagen type I molecules

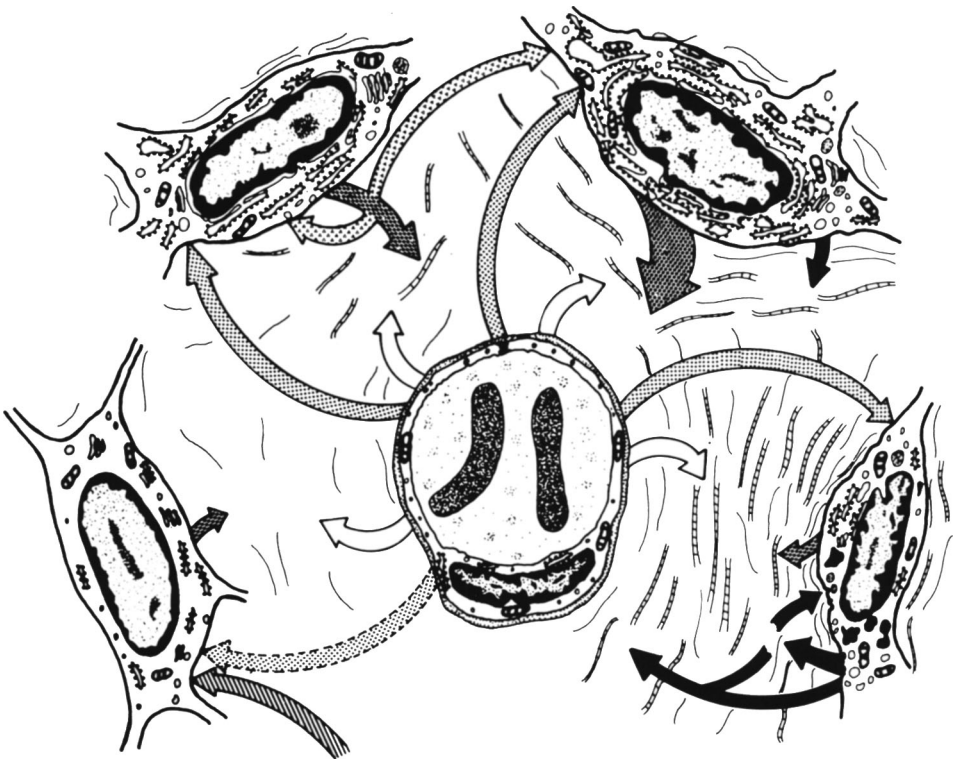

FIGURE 1. A scheme illustrating some selected aspects of cell-matrix interactions. As an example, a portion of a connective tissue is depicted. In the center of the diagram, a blood capillary can be seen. Around the capillary, cells of varying degree of differentiation are shown (from left to right: undifferentiated, differentiating, mature and aging cell). The space between the cells and the capillary is occupied by the extracellular matrix (the fibrillar components are masked by a few fine lines). Empty arrows: anabolic transport; dotted arrows: humoral factors regulating cell functions; arrows filled with circles: secretion of matrix constituents; arrows filled with triangles: effects of matrix on cell functions; dense arrows: matrix degradation and catabolic transport. (From Módis, L., *Acta Biol. Acad. Sci. Hung.*, 29, 197, 1978. With permission.)[4]

form the thick, interstitial collagen fibers (in skin, bone, tendon, etc.), while type III is present in a wide variety of tissues, generally associated with type I collagen, forming thinner fibrils (''reticulin'' fibers of the classical histology). Type IV collagen is specific to basement membranes, they form an orientad micellar network but not fibrils (see Chapter 7). Type V collagen shows a wide distribution. It is generally found in the pericellular matrix in form of small fibrils, and, therefore, it is frequently named as pericellular collagen and considered as a part of the exocytoskeleton.[13,17] Collagen type VI is present in a wide variety of tissues as interstitial collagen. Type VIII collagen is secreted by endothelial cells. Type XII collagen is expressed in embryonic tendon, but its role is still unknown.

Collagen may be involved in different interactions. Collagen molecules of the same type can be associated with each other forming fibrils or micelles (e.g., type I fibrils and type IV micelles). Different collagen types can also be bound with each other (e.g., in collagen fibrils of the hyaline cartilage[18]). Type VII — as a major part of anchoring fibrils — interconnects basement lamina to the underlying connective tissue matrix.[19] Different collagen types may be attached to the cell membrane via collagen receptors or by nectin molecules (fibronectin, chondronectin, laminin, see later). Proteoglycans also interact with collagen, predominantly by electrostatic forces but more specific interactions may also occur.[20] Considering these multisided interactions, collagens can be regarded as key molecules of the extracellular matrix organization.

B. ELASTIN

The elastic behavior of some tissues (lung, artery wall, skin) is mainly due to the presence of elastic fibers in the ECM. The morphological appearance of elastic fibers is simple, the mature fibers appear as homogeneous structures under electron microscope. They have been, therefore, considered to possess an amorphous structure. Polarization microscopy revealed that elastic fibers have submicroscopically oriented constituents.[21] These may correspond to the microfibrils of 10 to 12 nm in diameter recognized on the surface of developing and mature elastic fibers electron microscopically. The composition of the microfibrils is not yet well established because of the difficulties in solubilizing them.[22-24] It seems that microfibrils appear first in the extracellular matrix during the differentiation of the elastic fibers, and subsequently they are embedded in an amorphous component called elastin. The elastin composes about 50% of the mature elastic fibers. It is a rubber-like protein, rich in glycine, proline, and hydrophobic amino acids, and its conformation is like a random coil. Hydrophobic regions of the mobile spiral chains of elastin are held together by desmosine crosslinks. Between these crosslinks, the elastin molecules align when the fiber is stretched and recoil when the force is released.

Due to the technical difficulties encountered in elastic fiber research (e.g., the insolubility of the components), this component of the ECM is still the poor relative compared to collagen, proteoglycan, and glycoprotein biochemistry and morphology.

C. PROTEOGLYCANS

Proteoglycans (PGs) contain a protein core to which sulfated glycosaminoglycans (GAGs) (chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, heparin) are bound covalently. The only GAG which is not bound to a protein core is the hyaluronic acid. PGs represent a diverse group of glycoconjugates, with the only common basic feature that all of them contain at least one GAG side chain.[25-27] The core protein is generally linear in shape — except some globular domains — and the extended GAGs are perpendicularly oriented to the protein backbone. Due to these, larger PGs have a shape similar to a bottlebrush (for illustration, see Chapter 4). The GAGs have a helical conformation, and they — due to its high density of anionic charges (sulfates and carboxyls) can easily interact with microcations (Na^+, K^+, Ca^{2+}) and polycations (e.g., collagen).[4,20]

PGs can be classified according to (1) their size; (2) their GAG type; (3) their GAG content; (4) their core protein; (5) their abilities for interactions; (6) their localization (Figure 2). Based on these criteria, about 20 PGs can be distinguished. This structural diversity certainly implies considerable functional heterogeneity in the PG family.

PG molecules are located in different compartments of the ECM. One group is found at the cell surface. The best known example is the hepa n sulfate/chondroitin sulfate PG group. These PGs are either integral membrane proteins or they are attached to the cell membrane. They are involved in cell attachment by binding with their GAG chains to the GAG binding sites of matrix proteins such as fibronectin.[27] Another class of PGs is bound to collagen. Besides the electrostatic interactions of GAG side-chains of PGs and collagen, more specific protein-protein interactions also occur.[20,27] The most typical example is the dermatan sulfate/chondroitin sulfate PG (decorin) which has binding sites for collagen on its protein core.[27] In addition to ion and water binding as well as mechanical (space filling and shock-absorber) functions, PGs are shown to be involved in a number of biological processes such as cell differentiation and morphogenesis. The elucidation of their role in pathological processes like neoplastic phenotype expression and tumor progression also attracts more and more attention.[28]

D. NONCOLLAGENOUS PROTEINS

A large number of matrix glycoproteins have been isolated and characterized. Contrary to the PGs, their protein constituents bear only oligosaccharides and not GAGs. This family

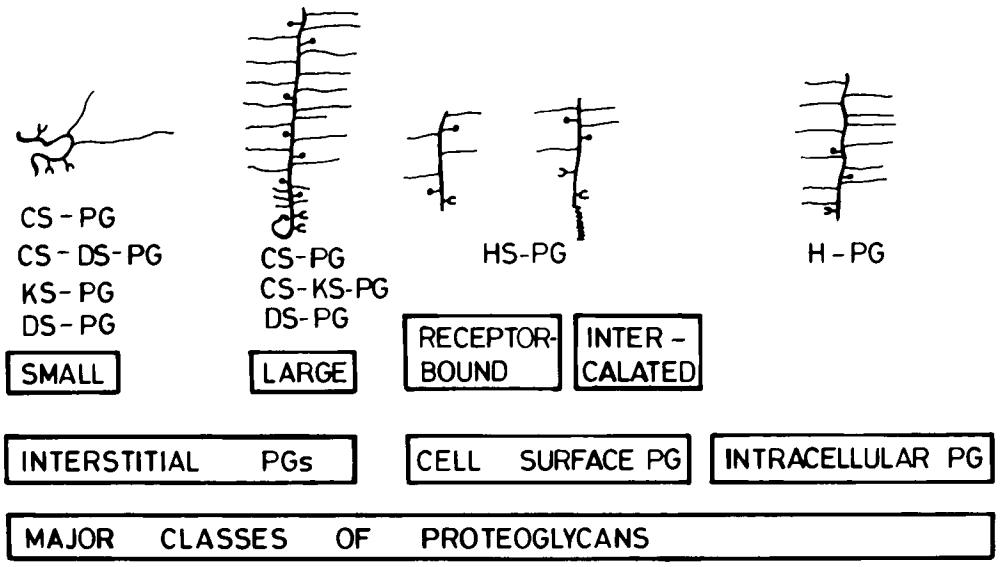

FIGURE 2. Schematic representation of the main PGs. CS: chondroitin sulfate; DS: dermatan sulfate; KS: keratan sulfate; HS: heparan sulfate; H: heparin.

comprises structurally and functionally very heterogenous macromolecules. They are involved in cell attachment and matrix organization.

The major cell-substratum adhesion molecules (fibronectin, laminin) are multifunctional and well-characterized glycoproteins.[1,7,29-33] They interconnect cell surface receptors (integrins) and collagen of the pericellular space. The cell binding domain of these molecules is a tripeptide recognition sequence Arg-Gly-Asp. Fibronectin is primarily synthesized by fibroblasts and endothelial cells and is involved — due to its multiple functional domains — in cell attachment, spreading, migration, differentiation, and matrix organization. Laminin is synthesized by epithelial cells and found in basement membranes. Like fibronectin, it also binds to other ECM components including collagen type IV, heparan sulfate proteoglycan, and nidogen/entactin (see Chapter 7). Chondronectin is supposed to bind collagen type II to the chondrocyte membrane.[34]

These molecules form a bridge between the pericellular matrix constituents (collagen and PG) and the cytoskeleton, because the microfilament compartment of the cytoskeleton is associated via a vinculin + talin complex with the cellular receptors (integrins) of these cell adhesive proteins (Figure 3, see also Chapter 9).

III. RECEPTORS INVOLVED IN THE CELL-MATRIX INTERACTIONS

Different cell-surface receptors have been identified which mediate cell-matrix interactions. The most important member of this receptor group is the integrin family.[35,36] Integrins consist of two subunits, and both of them have a large extracellular domain, a transmembrane, and a short cytoplasmic domain. The extracellular domain recognizes and binds the arg-gly-asp sequence of different matrix proteins including fibronectin, laminin, vitronectin, etc. The cytoplasmic domain interacts with the cytoskeleton via a talin-vinculin complex. Supposedly, this interaction mediates signal transfer from the ECM to the cytoplasm (and through a cytoskeleton-nuclear matric interaction to the nucleus).

Other receptors have also been identified, e.g., for hyaluronic acid[37,38] and collagen.[39,40]

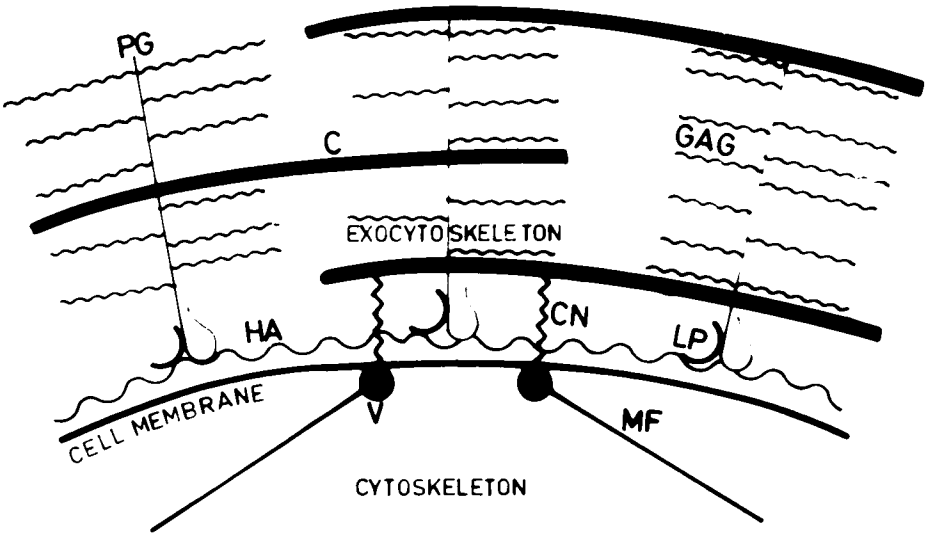

FIGURE 3. An oversimplified scheme showing the interconnection of cytoskeleton with the pericellular matrix (exocytoskeleton) in cartilage. PG: proteoglycan; GAG: glycosaminoglycan; C: collagen; HA: hyaluronic acid; CN: chondronectin; LP: link protein; V: vinculin; MF: microfilament. Receptors for collagen and hyaluronic acid are not shown. Instead of chondronectin, fibronectin binds collagen to integrin receptor of the cell membrane in other tissues.

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Chapter 2

PHYSICAL BACKGROUNDS OF POLARIZATION MICROSCOPY

TABLE OF CONTENTS

I Introduction 10

II. A Short Summary of Optical Principles 10

III. Glossary of Selected Terms for Polarization Microscopy 10

IV. Operations with the Polarization Microscope 25

Acknowledgment 26

References 26

I. INTRODUCTION

In this Chapter, an attempt is made to summarize the theoretical basis and the practical aspects of polarization microscopy. For more details, other works should also be consulted.[1-31]

First, we describe the most important optical principles. This short summary is supplemented with a glossary of selected terms used in polarization microscopy of biological specimens. Finally, some operations with the polarization microscope are also given for those who want to try this technique in ultrastructure research of biological specimens.

II. A SHORT SUMMARY OF OPTICAL PRINCIPLES

The oscillating electric field of linearly polarized light — generated by a polarizer — interacts with the valence electrons of anisotropic, birefringent, double refracting) material. The anisotropic material — due to its submicroscopically oriented structure — is characterized by two (in some crystals more than two) refractive indices (ordinary and extraordinary) perpendicular to each other. The state of polarization of light is influenced by this material. In the birefringent structure, the linearly polarized light splits into two components, the ordinary and extraordinary rays, which oscillate in two directions at right angles to each other and each at 45° with respect to the vibration plane of the incident polarized light and pass through the material at different velocities. Both beams will be slowed down inside the structure, but one (oscillating in the direction of the extraordinary refractive index) will be slowed down more than the other. There will be, therefore, a phase difference between the two beams. From the superposition of the two linearly polarized vibrations with a certain phase difference a resultant vibration is constructed after emerging from the birefringent specimen. The resultant vibration is generally an elliptical polarized light (may also be linear and circular), and its component parallel to the vibration plane of the analyzer — which is perpendicular to that of the polarizer — is transmitted by the analyzer. This light is observed in the eyepiece and used for qualitative and quantitative analyses of the birefringence.

The analysis of the ultrastructure at a biological specimen in polarized light is not limited to examination only between crossed polaroids. Some structures exhibit a direction dependent absorption of polarized light called dichroism. It can be quantified by cytophotometric measurements.

From qualitative and quantitative analysis of birefringence and dichroism, information can be indirectly obtained about the spatial orientation of asymmetrical submicroscopic constituents (molecules, micelles) of the specimen. Birefringent systems are extremely common in biology but some of them can only be recognized after special histochemical reactions which enhance the optical anisotropy of a structure. Classical 65polarization microscopy comprises techniques used mainly for unstained structures. Modern polarization microscopy is supplemented by a number of histochemical reactions which make this technique more powerful. The primary aim of this volume is to show the usefulness of the combination of histochemistry and polarization microscopy in connective tissue research.

III. GLOSSARY OF SELECTED TERMS FOR POLARIZATION MICROSCOPY

Analyzer — A polaroid containing highly oriented dichroic particles which transmit light with only one plane of vibration. It is inserted between the specimen and eyepiece in a polarization microscope. The plane of vibration of the analyzer is crossed to the plane of vibration of the polarizer when studying birefringence of a specimen. In this position, the light coming from the polarizer is extinguished. The microscopic field appears completely

dark, while birefringent specimens appear bright. The analyzer is removed when dichroism is observed. There are fixed and rotatable analyzers in use. Rotating analyzers are indispensable when measuring the retardation value of a specimen using a fixed quarter-wave plate.

Anisotropic material — A material whose properties are different in different directions. *Optically anisotropic materials* influence the state of polarization of the illuminating light because their structure is characterized by anisometric (asymmetric) submicroscopic particles oriented in two or three preferential directions. As the electronic resonators of this material are oriented in preferred directions, the material transmits light with different velocities in these directions, i.e., it shows two or three refractive indices. In biological specimens, there are generally two — ordinary (n_o) and extraordinary (n_e) — refractive indices and they are perpendicular to each other. Therefore, such materials are called birefringent (double refracting). The geometric symbol is the indicatrix or its cross section called index ellipse. Its long axis represents the n_e and the short axis the n_o.

Anomalous dispersion of birefringence — Variation of birefringence ($n_e - n_o$) with wavelength of the illuminating light. It is due to variation of the refractive index with wavelength (dispersion of the refractive index). The dispersion is normal if the two refractive indices (n_e and n_o) parallelly change, consequently the birefringence remains constant at different wavelengths. The dispersion is anomalous (anormal) if the two refractive indices change differently as a function of the wavelength of illuminating light. The ordinary refractive index (n_o) slightly changes (generally decreases towards the longer wavelengths), while the extraordinary refractive index (n_e) varies considerably with wavelength because more light is absorbed in the direction of n_e than in that of n_o. In this case, the birefringence changes as a function of the wavelength. If the dispersion curves of the two refractive indices cross each other at a certain wavelength (generally near to the absorption maximum of colored structures), vivid anomalous polarization colors appear in the polarization microscope and the sign of birefringence is different before and after the crossing point.

Anomalous dispersion of birefringence appears in highly oriented light-absorbing structures. It can be investigated by plotting curves of anomalous dispersion of retardation in the following way. Retardation values of a birefringent (and generally stained) material are measured with compensator plates at different wavelengths. Retardation values (Γ) are plotted against the wavelength (λ). As for

$$\Gamma = d(n_e - n_o)$$

where d = geometric thickness of the structure measured which was constant during the measurements, the curve obtained shows the anomalous dispersion of birefringence. The shape of the curve reflects the degree of orientation of the structure: the steeper the curve, the greater the degree of orientation. Examples are shown in Chapter 3.

The anomalous optical rotatory dispersion (ORD) is the dependence of rotary birefringence on wavelength. If the sign of birefringence is also changed (near to the absorption maximum) this phenomenon is called the Cotton effect. If the sign of birefringence is positive at longer wavelengths, the Cotton effect is positive, and in a reversed situation it is negative. Intrinsic Cotton effect is a property of chiral molecules, while extrinsic Cotton effect is induced by reagents (e.g., dye molecules) bound to the molecule under investigation. Curves of anomalous dispersion of birefringence and ORD curves may be similar in the same material.

ORD is a stereochemical method to elucidate molecular conformations. Using this technique, the helical conformation of different glycosaminoglycans has been described.[32-38] X-ray diffraction studies have also revealed the helical conformation of these macromolecules.[39-43]

ORD of cells and tissues can also be studied by a microspectropolarimeter and it is suggested that conformational changes of intracellular biopolymers may be detected with this method.[44,45]

Anomalous polarization color — This is produced by the orientation of light — absorbing bonds (e.g., dye molecules), and it is detected by examining the specimen between crossed polaroids and varying the compensation by rotating the compensator plate. A given amount of compensation will only accomodate certain wavelengths. Light of other wavelengths is uncompensated and the specimen appears colored. For example: it is known that congo red stains amyloid and enhances its optical anisotropy very strongly. Congo red molecules absorb green light, therefore, the dye molecules deposited on amyloid fibrils in an oriented manner are strongly birefringent in green but weak in blue and red light. Thus the polarization color of the amyloid stained with congo red is green. After additive compensation in white illuminating light, yellow anomalous polarization color appears, while after subtractive compensation greenish blue anomalous color can be observed. Another example concerning toluidine blue will be shown in Chapter 3. This dye stains polyanionic structures in a metachromatic red color. Due to the ordered (generally axiperpendicular) deposition of the dye molecules, toluidine blue enhances the birefringence of the structure stained. The metachromatic toluidine blue aggregate absorbs green light, therefore it is strongly birefringent in green. Its polarization color is, therefore, green. After additive compensation, light of longer wavelength (orange), and after subtractive compensation, light of shorter wavelength (blue) appears in the structure. Knowledge of anomalous polarization colors helps greatly if we want to describe the orientation patterns of dyes bound to oriented biological structures. If the geometry of dye-binding to the structure is known, the ultrastructure of the biological specimen can be inferred from the dye orientation pattern.

Artifacts in polarization microscopy — Two kinds of artifacts can be distinguished. First, there are foreign materials exhibiting optical anisotropy (paraffin residues, dust, talc, textile and paper fibers, dye precipitates, other birefringent dirts) whose presence in the microscopic specimen disturb the polarization microscopic analysis or microphotography.

For histologists, the presence of paraffin is especially important. Due to the thermic orientation of the molecules, cooled (solid) paraffin is highly birefringent and melted paraffin is isotropic. Insufficiently deparaffinized sections contain small birefringent particles — especially in cell nuclei[46-48] — which may cause an incorrect interpretation. Paraffin residues can be recognized by a simple probe: the specimen is warmed up to 55 to 60°C and immediately checked in polarization microscope. If the birefringence disappears after warming and gradually reappears during cooling on the microscope stage, the birefringence is caused by paraffin. The complete removal of paraffin from the embedded material is, therefore, essential before polarization microscopic investigation. Generally, a prolonged deparaffinization in xylene at 37° or 56°C (4 to 24 h, depending on the type of paraffin) or in an 1:1 mixture of methanol-chloroform at room temperature (2 to 12 h) must be carried out.

It is of the same importance to keep any kind of microscopic preparations as well as the optics of the microscope free of dust.

The second group of polarization microscopic artifacts comprises the cases of pseudoisotropy (see under pseudoisotropic materials).

Biaxial crystals — These have three different refractive indices: n_α (lowest), n_β (middle) and n_γ (highest). Its geometric symbol is a triaxial (general) ellipsoid with three unequal principal axes. Such crystals have two optical axes (i.e., there are two positions where the material seems to be isotropic). Both optical axes are in the plane of the smallest and the largest refractive index. Biaxial materials are rarely met in biological structures.

Birefringence — Birefringence or double refraction is an intrinsic property of a material, and is a modification of polarized light by a structurally and optically anisotropic material.

It can be observed between crossed polars if the plane of vibration of the polarizer and the reference axis of anisotropic material are at 45° to each other. Birefringence (Δn) can be described by the following equation:

$$\Delta n = n_e - n_o$$

where n_e and n_o are the extraordinary and ordinary refractive indices, respectively. The linearly polarized illuminating light splits into two components which oscillate in the two directions of the refractive indices.

Major categories of birefringence are as follows: intrinsic (crystalline), form (textural), strain (tension or accidental birefringence, accompanied by a photoelastic effect), rotary (optical rotation), flow, and electric birefringence. In many cases, these categories may be combined. Filamentous molecules (e.g., hyaluronic acid)[49,50] exhibit flow birefringence. Electric birefringence (Kerr effect) is due to the orientation of the solute molecules as the solution is subjected to a pulsed electric field.[51] This is a sensitive indicator of molecular conformation changes and associations. A prolate ellipsoid shape and a 1.8 ± 0.1 μm length of the proteoglycan aggregate, characteristic for the cartilage matrix, were inferred from electric birefringence studies.[52]

Circular polarized light — If the electric vector of a circularly polarized light beam is projected on a plane normal to the axis of propagation, the projection of the tip is a circle. It is produced by birefringent materials showing λ/4 and 3/4λ retardation (Figure 1). Circular polarized light is sometimes used as illuminating light in the polarization microscope. Its advantage over the use of linear polarized light is that birefringence of the structure can be observed in any direction including the vibration planes of the polarizer and analyzer where no birefringence can be seen using linear polarized illuminating light. This technique is useful in investigating both spherical (e.g., cell membranes) and fibrous structures [53,54] (Figures 2,3). In practice, two λ/4 compensator plates are necessary for microscopic investigations in circular polarized light. One plate is inserted between the polarizer and specimen; the other plate is placed between the specimen and eyepiece. The n_γ directions of the plates should be crossed with each other, and the n_γ direction of the lower plate be at 45° with respect to the plane of vibration of the polarizer.

Compensation — Operating with a compensator plate of the polarization microscope, we can establish the sign and retardation value of birefringence a structure. It is carried out by rotating the compensator (or in the case of fixed compensator plate, by rotating the analyzer) until the birefringence of the spencimen is extinguished (compensated). When the higher refractive indices of the compensator plate and specimen are crossed, they will subtract (subtractive position). This is the generally used position for measurements. When the direction of higher refractive index of the compensator plate runs parallel to the direction of higher refractive index in the specimen, their effects will be added (additive position). Unstained or colorless specimens are generally compensated in white or green light, while colored (stained) biological specimens are compensated in appropriate monochromatic light.

Compensators — Birefringent plates of known retardation are used to determine the sign and retardation of birefringence. The direction of their slow ray (n_γ) is usually marked with γ. Compensator plates are inserted between the object and analyzer.

There are different types of compensator plates. The wedge compensators are used in cystallography. The tilting compensators (Berek and Ehringhaus plates) of great measuring range (retardation of several thousand nm) are not accurate enough for the measurement of biological structures. Compensators of fixed azimuth generally belong to the standard equipment of a polarization microscope. One of them is the "red I order plate" (its retardation value is one full wavelength), and it is used to estimate the relative sign of birefringence of colorless structures of greater retardation.

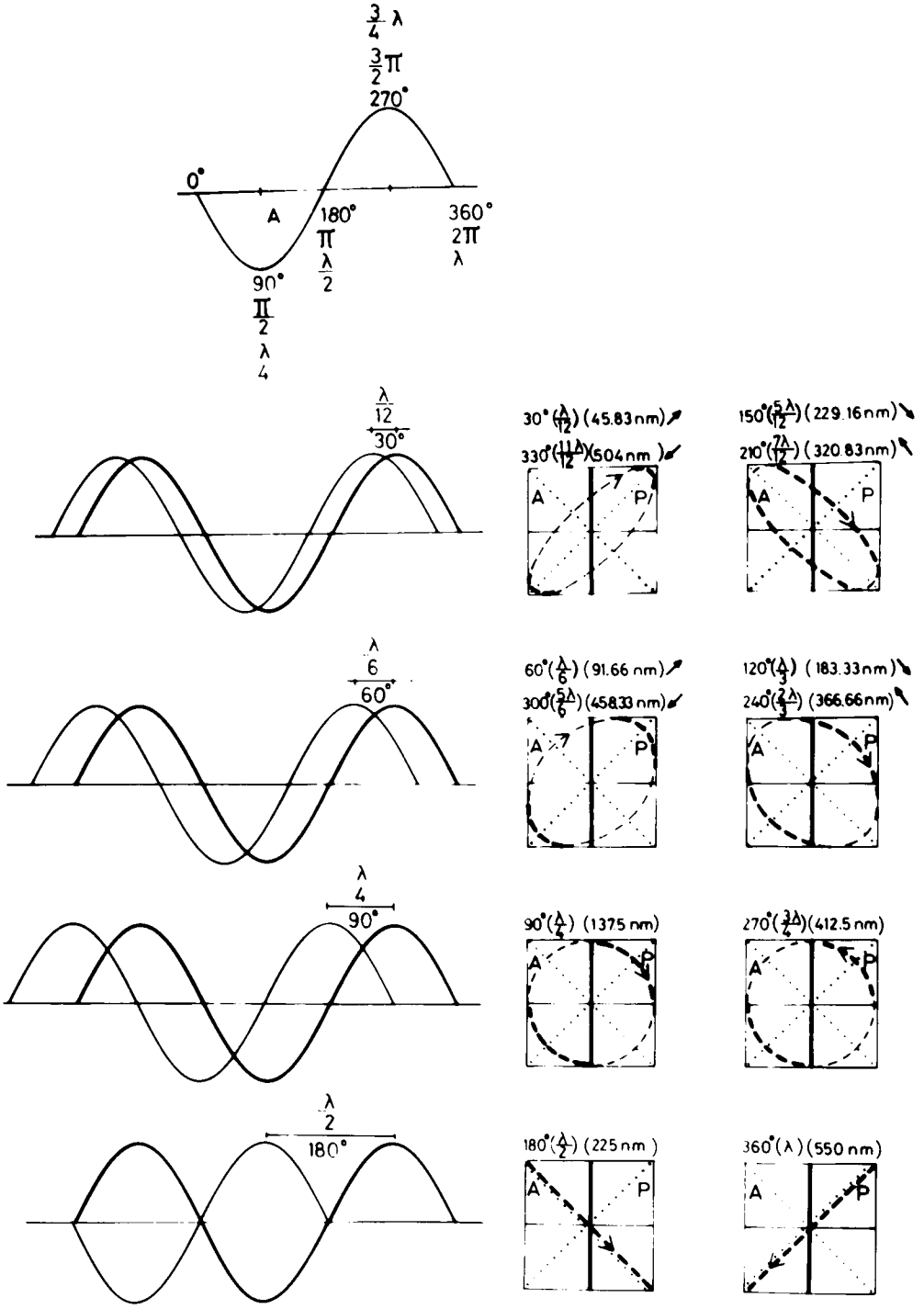


FIGURE 1. Schematic diagram showing different retardations (on the left) and different types of the polarized light (on the right). The right sets of diagrams represent projections on a plane perpendicular to the axis of propagation of the light. A, B = axes of analyzer and polarizer, respectively. Retardation (Γ) is expressed in fragments of wavelengths, in nm, and in phase angles. Note that a retardation of $\lambda/4$ results in circular polarized resulting light. If $\Gamma = \lambda/2$, the resulting beam will be a linearly polarized light. Elliptically polarized light is obtained for other phase differences shown in the Figure.

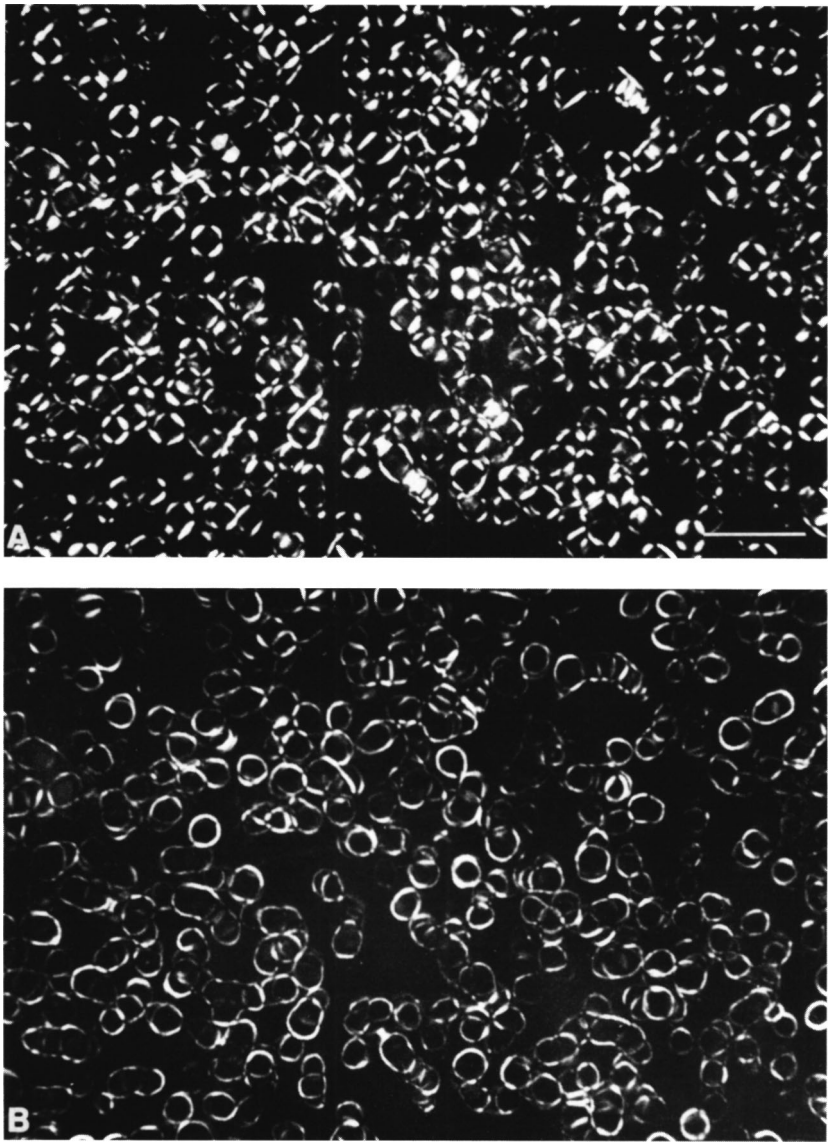


FIGURE 2. Human peripheral blood smear stained with toluidine blue at pH 7.0 and taken in linear (A) and circular (B) polarized light. Note the birefringence of the cell membranes of the erythrocytes. Using linear polarized light, the cell membranes appear as Maltese crosses. Birefringent membranes appear as continous bright circles when illuminated with circular polarized light. Scale: 30 μm.

The other one is the quarter-wave plate (λ/4) which is widely used for measurement of retardation values according to the Sénarmont method[20] and estimation of the relative sign of birefringence. Using this method, the λ/4 plate is inserted with its n_γ in subparallel position below the analyzer, and the analyzer is rotated until the birefringence is extinguished (the specimen becomes dark). The retardation value of the birefringent structure (Γ) is calculated as follows:

$$\Gamma = \frac{\alpha \times \lambda}{180}$$

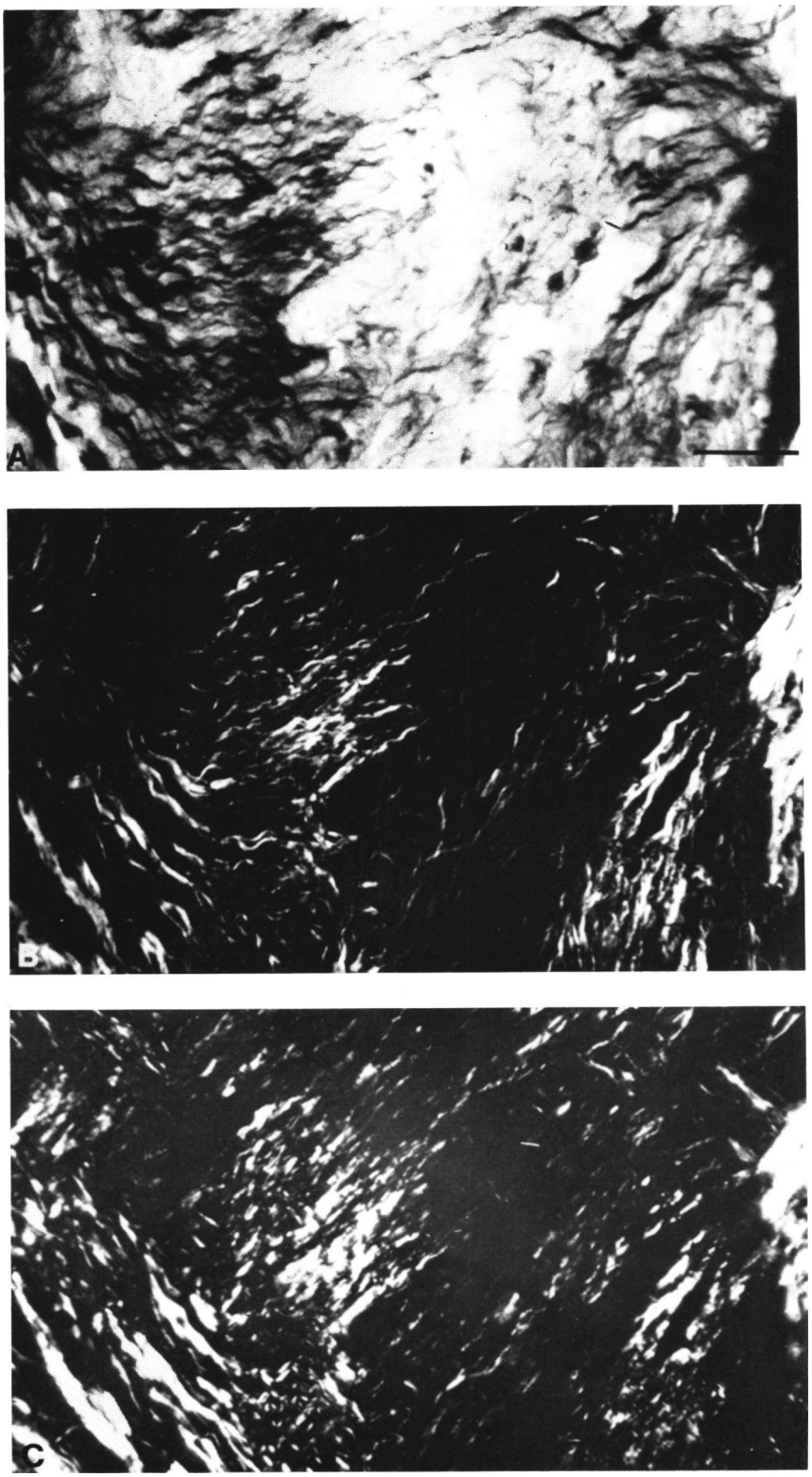


FIGURE 3. Collagen fibers in pericapsular region of knee joint of a 4.5-month-old human fetus stained with picrosirius red F3B and taken in normal (A), in linear (B) and circular polarized light (C). Scale: 100 μm.

where α = an angle reading of the rotating analyzer in extinction position, λ = wavelength of the illuminating light.

Two quarter-wave plates are needed if polarization microscopic investigations are carried out in circular polarized light (see under this key-word).

Goldstein[55] proposed a new fine analytical procedure for transparent, birefringent, and dichroic specimens to measure separately the retardation and dichroism. In this elegant technique, which is a further development of the classical Sénarmont method, two quarter-wave plates (one fixed and one rotatable) are inserted between the specimen and the rotatable analyzer.

For accurate measurement of small retardation values (0.5 to 1.0 nm), Brace-Koehler rotatable compensators are used. Their retardations are generally $\lambda/10$, $\lambda/20$, $\lambda/30$. They are used for measurement as follows: The polarizer and analyzer are crossed. The reference axis of the specimen is oriented at $\pm$ 45° to the polarizer. The slow axis (n_γ) of compensator is oriented parallel to the vibration plane of the polarizer. The compensator is rotated until the specimen is extinguished and the angle (α) is read from the drum vernier linked to the rotating mica plate. The retardation value of the specimen (Γ) is then calculated as follows:

$$\Gamma = \Gamma_\kappa \times \sin 2\alpha$$

where Γ_κ is the maximal retardation value of the compensator plate at the wavelength of the illuminating light (this value is provided by the manufacturer).

Combination of the polarization microscope with a cytophotometer has been suggested to increase the accuracy of measurement of the retardation with compensator.[56]

Although, measurement with compensator plates is relatively simple, it is a time consuming and tiring technique and requires well-trained eyes of the microscopist. There is also a serious operational problem: the microscopist is required to make a rapid change in both visual accommodation and dark adaptation in the transition from viewing through the microscope to reading the drum vernier. To overcome these difficulties, an attempt has been made to construct a computer-compatible system to measure and record retardation.[57,58] The system consists of a high precision potentiometer linked directly to the drum vernier of a compensator plate or analyzer. A highly stabilized fixed voltage is applied to the potentiometer and the voltage difference between the potentiometer wiper and one end of the resistance element is directly proportional to the compensator angle. This voltage is converted to a digital time base and stored for subsequent computer analysis. Recently, a computer-compatible digital compensator called Retarmet has been developed by Carl Zeiss (Jena, GDR) for the Jenapol polarization microscope of the same factory. It is hoped that these modern compensators will be widespread and will greatly facilitate quantitative polarization microscopic investigations with their simplicity and great accuracy.

Composite (mixed) bodies — Idealized textures containing oriented asymmetrical units (rods, layers, platelets or discs) which can be regarded as models of biological structures exhibiting optical anisotropy.[4,12,28,56,59] The units are small in diameter with respect to the wavelength of light. If their units are isotropic, the composite bodies exhibit form (textural) birefringence. According to the theory of Wiener,[59] the rods with an index of refraction n_1 are dispersed in a homogeneous medium with refractive index n_2, and all of them are oriented parallel to the optic axis. In this case, the sign of form birefringence is positive with respect to the optic axis. From biological structures, the skeletal muscle fiber and the sperm tail approximate this type of composite bodies. In other types of composite bodies, layers or discs are perpendicularly oriented with respect to the optical axis. In this case, the sign of birefringence is negative with respect to the optic axis. Form birefringence is frequently combined with intrinsic birefringence in biological structures. In this case, the units of the composite body possess an oriented molecular structure. For example, the collagen fibers

show positive form and positive intrinsic birefringence, and cell membranes exhibit positive form and negative intrinsic birefringence (with respect to the tangent of the cell). Form and intrinsic birefringence can be distinguished by analysis of the imbibition curves.

Dichroic dyes — These are deposited on a structure in an oriented manner thereby modifying the optical properties of the structure (birefrigence, dichroism). Generally, dyes with asymmetrical structure and planar configuration comprise this group. The absorption of polarized light by such dyes is direction-dependent. If the plane of the light vibration is parallel with the long axis of the oriented dye molecules, the absorption is stronger when compared with a perpendicular position. The material stained with a dichroic dye appears, therefore, to vary in color when observed in polarized light of different vibration directions. There are some naturally occuring structures containing dichroic dyes (e.g., feathers of some birds). The great majority of these dyes, however, are used for staining of tissues (e.g., congo red for amyloid, toluidine blue for glycosaminoglycans, sirius red F3B for collagen; for more details see Schmidt,[60] Ruch,[61] Missmahl).[62]

Dichroic staining — An oriented array of dye molecules are deposited on a structure during this procedure which results in the modification of the optical properties of the structure stained. Its synonyms are the topochemical reactions[6] and topo-optical reactions.[63] In this volume, the term topo-optical reaction will be used. Dichroic stainings have been used for a long time.[2,60,64] The recent progress of the polarization microscopical techniques used in biology can be attributed to the development of new dichroic stainings among which a number of histochemical reactions are also found by means of which dichroism can be induced.

Dichroism (pleochroism) — Anisotropic absorption of polarized light. In linear dichroism, the absorption varies changing the azimuth of transmitted linear polarized light. Certain crystals and molecules absorb different wavelengths of polarized light to different degrees depending on the relation of the plane of polarization to the optic axis of the dichroic material. Due to this, different colors may be produced when transilluminating the material in different directions with polarized light. In circular dichroism (CD), the absorption of circular polarized light changes upon reversing the direction of rotation of the transmitted light. CD is a valuable tool in stereochemistry to describe molecular configuration.

Form and intrinsic dichroism can be distinguished (see the theory of composite bodies). Form dichroism is shown by structures in which submicroscopic elongated units are oriented in a preferential direction. Intrinsic dichroism is a property of the molecules themselves. Depending on the absorption of the material, dichroism can be observed in the visible spectral range and in infrared or ultraviolet light. For example, oriented filaments of DNA exhibit a negative dichroism at 260 nm which is due to the perpendicular orientation of purine and pyrimidine bases to the long axis of the molecules.

The sign of dichroism may be positive and negative. A material exhibits positive dichroism related to its preferential axis if it shows maximum absorption of polarized light vibrating parallel to the direction of the preferential axis. Amyloid fibers stained with congo red or collagen fibers stained with sirius red F3B show positive dichroism. The sign of dichroism is negative with respect to some distinguished axis of the material if the absorption is maximal in a direction perpendicular to that axis. For example, collagen fibers or cell membranes stained with toluidine blue show negative dichroism (in the case of cell membranes the reference axis is the tangent of the cell). In both of the latter examples, the negative dichroism is due to axiperpendicular orientation of the dye molecules to the structures.

Dichroism occurs naturally in some tissues containing colored pigments, or it can be induced by appropriate staining techniques. Dichroism provides information on the submicroscopic orientation of the structure, and it is a powerful tool to demonstrate dye orientation in the tissue. For microscopic demonstration of dichroism, the specimen is placed

above the polarizer and observed without an analyzer. To detect linear dichroism, the azimuth of linear polarized light is varied and color changes are observed. It can be measured by a cytophotometer: absorption curves are taken from different planes of the illuminating polarized light (perpendicular and parallel with respect to the direction of a preferential axis of the structure). The intensity of dichroism can be calculated by subtracting the extinction values obtained at perpendicular position from that of those measured at parallel position:

$$\Delta E = E_{\parallel} - E_{\perp}$$

Circular dichroism is examined by illuminating the specimen by circular polarized light produced by a quarter-wave plate placed just above the polarizer in a 45° position. The plate is rotated through an angle of 90° and the color changes of the object are observed. For more details concerning dichroism see other references.[18,20,28,61,62,65-67]

Dispersion of birefringence — This is the variation of birefringence with wavelength (λ), and it is a consequence of the dispersion of the refractive index (n). The dispersion is normal if n decreases with λ, and abnormal if n increases with λ. The formula of the dipersion of birefringence (D):

$$D = \frac{n_{e1} - n_{o1}}{n_{e2} - n_{o2}}$$

where n_{e1} and n_{o1} are the refractive indices at short wavelength, and n_{e2} and n_{o2} are the refractive indices at long wavelength.

Elliptically polarized light — The terminal points of the electric vector in a propagated ray rotates in a path which can be projected as an ellipse on a plane normal to the axis of propagation (Figure 1).

Extraordinary ray — When a light beam enters birefringent material it is split into two plane polarized beams. Their vibration planes are perpendicular to each other. One beam obeys the ordinary laws of refraction, and this is called ordinary ray. The velocity of this light beam is maximum. The other ray is designated as the extraordinary ray, and its velocity is minimum. It does not obey the ordinary laws of refraction. The refractive index for the extraordinary ray is n_e, and it is parallel with the major axes of the molecules forming the anisotropic structure (= slow axis). The extraordinary ray is retarted because this light interacts with chemical bonds.

Fluorescence polarization — Fluorescence emitted by dye molecules bound in an oriented manner to a structure is highly polarized because of the dichroic nature of the molecules (fluorescence polarization). If the molecules rotate in the period between absorption (excitation) and emission (it takes about 10^{-8} s), the fluorescent light is depolarized. The depolarization can be measured.[68,69] The depolarization is related to the rotational diffusion constant of the dye molecule. If such dyes are bound to a macromolecule, from the depolarization of the fluorescent dye the rotational diffusion constant of the macromolecule can be obtained. This technique is mostly used for analysis of particles in solution.[69,70] The application of fluorescence polarization for the investigation of submicroscopic orientation in cell and tissue structure is also possible although not widely used.[71-81]

Form (textural) birefringence — This is found in mixed bodies wherein asymmetrical particles (rodlets, platelets) of a given refractive index are dispersed in an oriented manner in a medium of different refractive index. Form birefringence can be abolished by replacing the medium with one having a refractive index equal to that of the dispersed bodies. This is the basis of the imbibition analysis.

Imbibition analysis — This is a classical and well established method of polarization microscopic ultrastructure research. Unstained structures are mounted in media of different

refractive indices, the retardation values of the birefringence are determined in each medium, and they are plotted against refractive indices. The curves obtained are called form birefringence curves. If there is a distance between the site of minimum retardation value of the curve and the abscissa, the structure also has intrinsic birefringence (Figures 4, 5) (see also Chapter 12). It is a useful and sensitive technique to show structural alterations of the collagen fibers.[82,83] Using this method, Vidal[84] was the first to show the axiparallel orientation of sulfated GAGs to the collagen fibers in unstained tendons. A similar orientation pattern of sulfated GAGs has been revealed by imbibition analysis in cartilage matrix[85] (Figures 4, 5).

Intrinsic (or crystalline) birefringence — This is due to asymmetrical alignment of chemical bonds or molecules, and it is an inherent property of the structure. The intrinsic birefringence does not vary in the function of the refractive index of the mounting medium. For this reason the two contributing factors of birefringence, form and intrinsic birefringence, can be separated from each other by imbibition analysis.

Isotropy — This occurs in isotropic materials where a large number of electronic resonators are distributed throughout a medium in a perfectly random manner. Isotropic materials do not influence the state of polarization of light, they are optically homogeneous, and can be characterized by one refractive index.

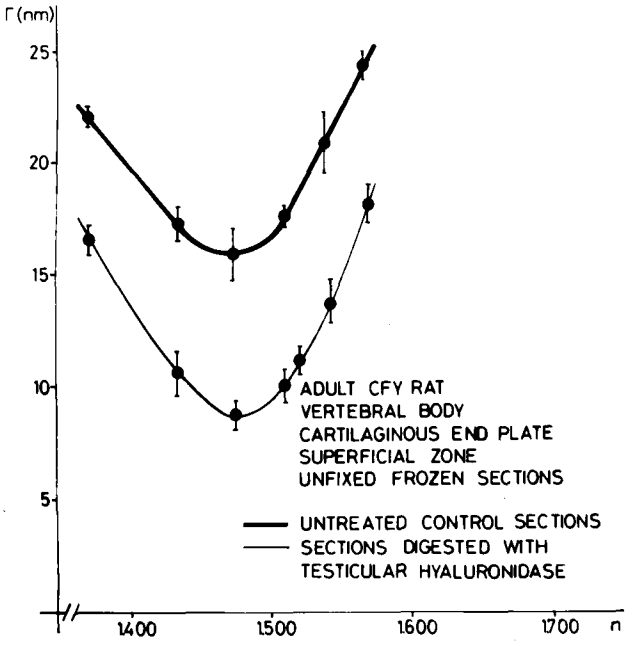
Linear (plane) polarized light — The electric vector of the light wave oscillates in a single plane. It is produced by structures where the $\Gamma = n\lambda$, where n = 1,2,3, etc.; or $\Gamma = n\lambda/2$ where n = 1,3, etc. If $\Gamma = n\lambda$, the resultant beam oscillates parallel to the polarizer axis and it is not passed by a crossed analyzer (Figure 6). The birefringence of such structures cannot be seen. Contrary to this, the resultant beam produced by a retarding object of $\Gamma = n\lambda/2$ oscillates parallel to the analyzer axis and it passes the analyzer with maximum brightness (Figure 6). Linear polarized light is produced by the polarizer in a polarization microscope.

Micelle (micella) — This term was introduced by Nageli in 1858 (see Reference 12) to describe long, submicroscopic colloid particles of crystalline structure. Micelles are supramolecular units, more often packets of chain molecules in parallel arrangement. Micella is a diminutive of the latin mica, a crumb.

Optic axis — This is a direction parallel to which a linear polarized light can be propagated with equal transmission velocities for all azimuths of polarization of the electric vector of the beam (e.g., the long axis of a collagen fiber). If light arrives in this direction, no optical anisotropy can be observed. The optic axis can be determined by finding an orientation in which the birefringent structure appears dark at all angles of rotation of the stage between crossed polaroids. In this position the optic axis of the specimen is parallel to the axis of the optical system of the microscope. The orientation of the optic axis of uniaxial materials may be longitudinal or transverse with respect to the long axis of the structure. The former is a positive uniaxial body (e.g., a collagen fiber), and the latter is negative (e.g., a collagen fiber after phenol reaction). In addition, the optic axis may be radial, circumferential, or tangential, and helical or spiral.

Ordinary ray — One of the split polarized light beams in a birefringent material. It oscillates in a plane perpendicular to the extraordinary ray (fast axis of transmission). The velocity of this light beam is maximum, and not retarded.

Polarization colors — They may appear in unstained, colorless or stained birefringent structures. Polarization color appears in an unstained specimen if one spectral region of the transilluminating white light is missing. In this case the complementary color to the missing wavelength is seen in the polarization microscope. If we see, for example, a purple color in an unstained structure, we know that the retardation value of this structure is about 550 nm. In this structure the green light (λ = 550 nm) is linearly polarized and it will not be transmitted by the analyzer, and we see the complementary purple color (= "red I").



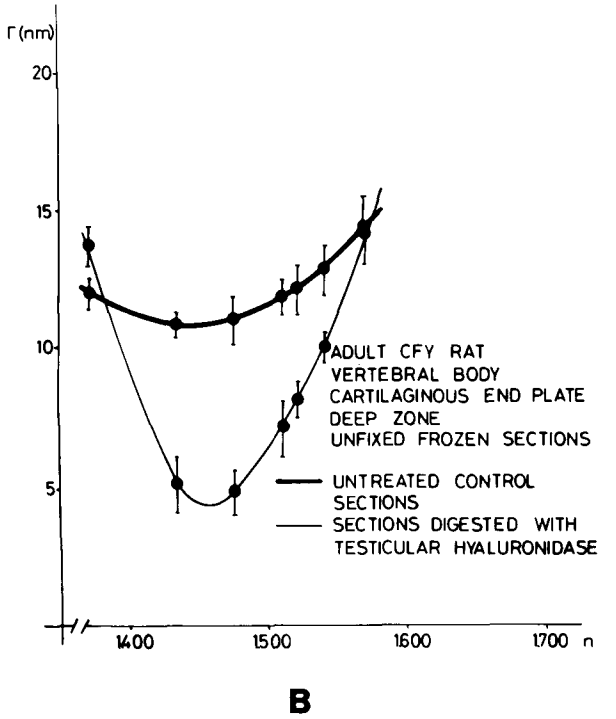
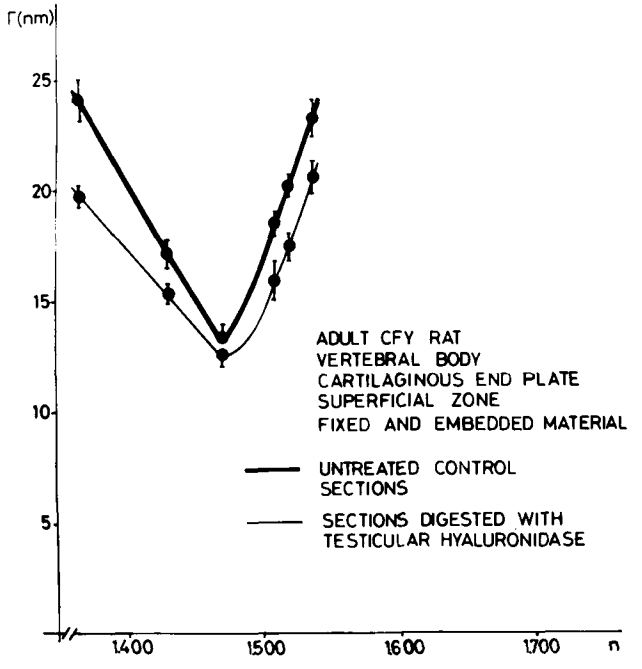


FIGURE 4. Form birefringence curves of unfixed cartilage matrix taken after an imbibition analysis carried out without and after digestion with testicular hyaluronidase. The decreased retardation values obtained after the digestion clearly indicate that sulfated GAGs are also oriented in the cartilage matrix. These molecules, or at least a population of them, are arranged parallel to the collagen fibers.



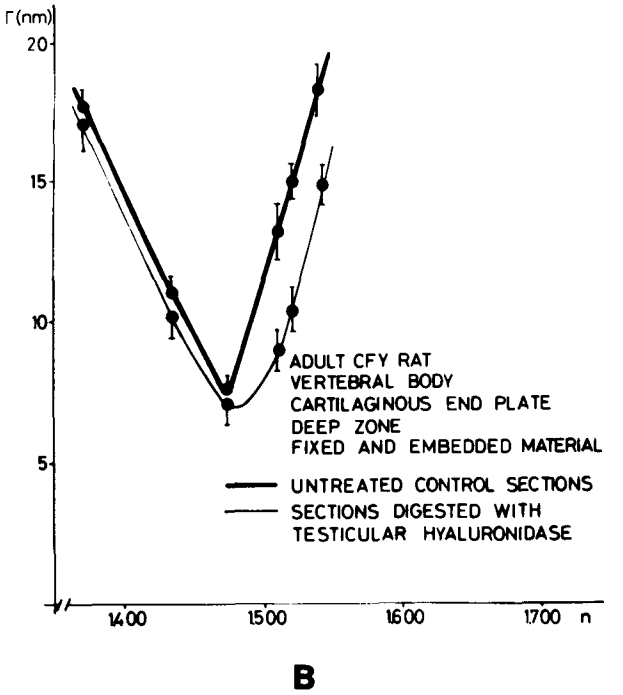


FIGURE 5. Form birefringence curves recorded from the extracellular matrix of cartilage fixed and embedded without and after digestion with testicular hyaluronidase. The digestion did not significantly alter the form birefringence curves indicating that unfixed specimens are better for imbibition analysis of the GAG molecules (compare with Figure 4).

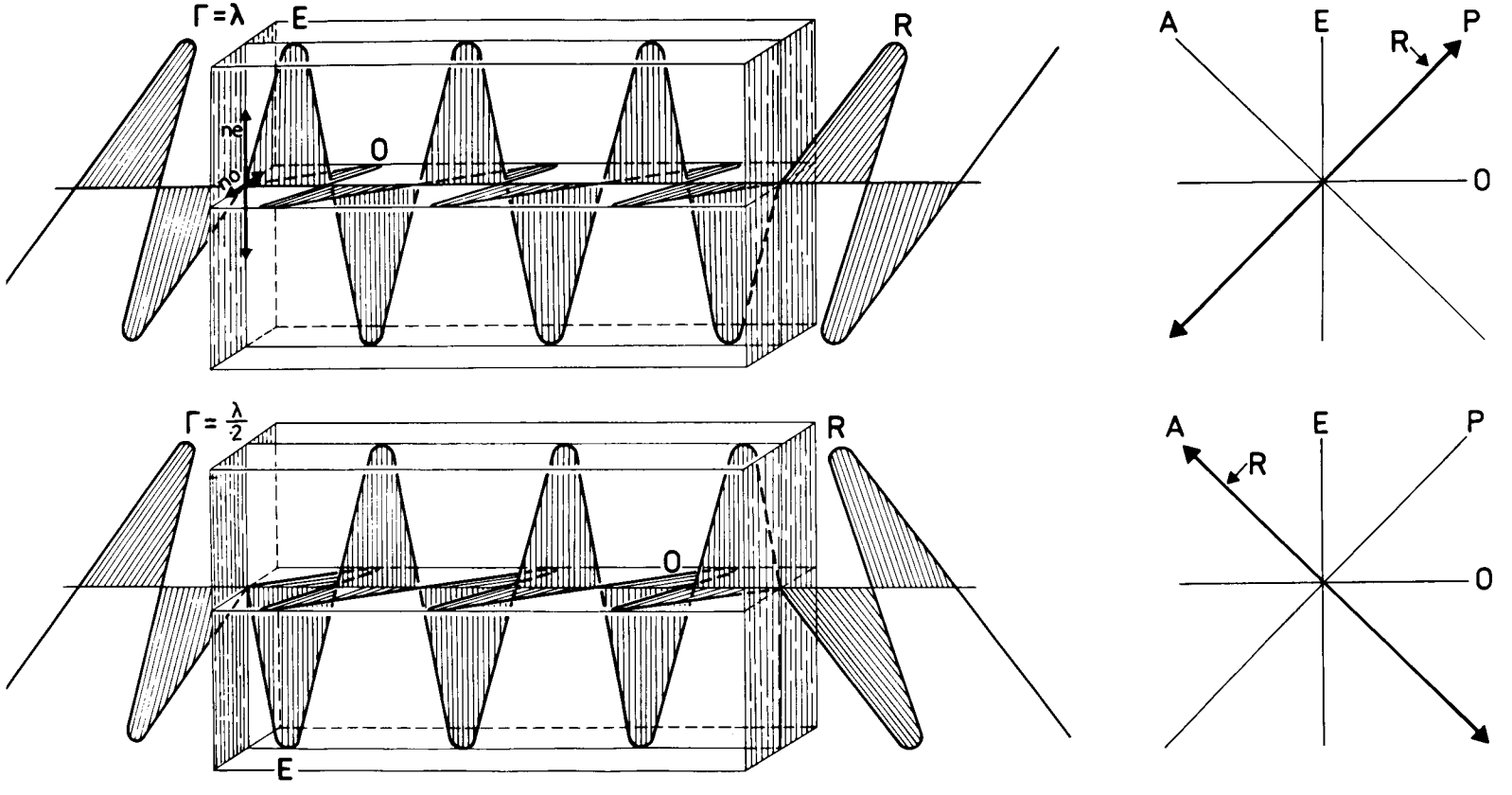


FIGURE 6. A scheme showing two birefringent materials. The upper one has a retardation value $\Gamma = \lambda$ with $\Gamma = \lambda/2$ in the lower one. A linear polarized light enters the structure (on the left) and splits into two components, the extraordinary (E) and the ordinary (O) rays, which oscillate in two directions at right angles to each other. The resulting beam (R) is linear polarized light in both cases, but it cannot pass the analyzer if $\Gamma = \lambda$. On the right side of the figure, the vibration directions are shown on a plane perpendicular to the axis of propagation of the light. A, P = axes of analyzer and polarizer, respectively; n_e, n_o = extraordinary and ordinary refractive indices of the birefringent structure.