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SPECIAL ASPECTS IN THE STUDY OF CONNECTIVE-TISSUE FIBRES IN POLARIZED LIGHT

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Lately, modern morphology and biology are making increasing use of polarization optics as a means of research of submicroscopic structures (1, 8, 13, 18, 19). The literature dealing with the optics of polarized light is for the major part concerned with the use of the polarizing microscope in crystallography (5, 6, 10, 12, 14), a domain of science in which much had been achieved with this means of observation by the time medical science and biology began to take deeper interest in it. Laws established in crystallography were being adopted and adapted, and a start was made to apply imbibition methods in quantitative analysis. Since, then, several fundamental works have been published treating of the applicability of the basic principles of polarization optics in medicine and biology. Among Hungarian authors, ROMHÁNYI [15, 16, 17] merits to be mentioned in the first place, who has strongly proclaimed the outstanding significance of the optics of polarized light in the submicroscopic study of connective-tissue fibres.

The present paper is an attempt to decide which fundamental laws can be taken over from crystallography and which need to be revised. For there are many bearings and guiding principles which if disregarded are likely to involve the risk of erroneous conclusions when studying biological objects under the polarizing microscope. Unlike to inorganic crystals, to biological objects the imbibition method cannot be applied unconditionally since many of the imbibition liquids react with them. Disregard for this very fact being responsible for a number of erroneous conclusions in the literature, it is proposed to deal with them briefly in this communication. Today, the electron microscope, the X-ray diffraction apparatus, and other modern procedures of observation afford a means of checking results obtained by polarization microscopy. The points to be made are best illuminated with the behaviour of connective-tissue fibres, collagen fibres in particular, since being objects of optically arranged structure they have readily definable double refraction.

Intensity of double refraction. Thickness of sections

Wavelength and velocity of the two rays emerging from the test objects differ from those of the incident ray. The interference colours from which it is possible to infer as to the intensity of the double refraction, result from the phase difference. The intensity of the double refraction is influenced by the thickness of the test object ; it changes in direct proportion to layer thickness. E. g., a 100 μ thick section from a birefractive crystal displays a double refraction twice as intensive as a section 50 μ in thickness. This law generally applies to biological objects, yet occasionally double refraction increases with decreasing layer thickness. The explanation for this is that if, on preparing the section, one layer, anisotropic in itself, separates from the other above or beneath it, which too is anisotropic but of different orientation, its double refraction is brought more into prominence. This is encountered whenever in the test substance the particles responsible for anisotropy are not of the same orientation in all the layers ; it is frequently observed in connective-tissue fibres following different courses. These, then, are the cases in which the paradoxical phenomenon may manifest itself, in which decreasing layer thickness is accompanied by increasing double refraction (Fig. 1).

Another phenomenon is the swelling of the test substance, in our case of collagen, in consequence of chemical pretreatment (ascorbic acid) to, not infrequently, 50 times its original thickness. When this is the case, double refraction must be accepted at its measured value, and not reduced to its 1/50th on account of the increase in layer thickness, as else error would be piled on error since in relation to native fibres the double refraction of swollen fibres decreases markedly, anyway (Fig. 2).

The situation is different in crystallography where the intensity of the double refraction is often observed to increase with the growth of the crystal in the same proportion as the particles crystallizing from the solvent add to the size of the crystal.

In view of these considerations the right procedure to follow is to work with quantitatively relative values. This means that we begin to work with preparations of identical thickness and accept the quantitative double refraction values obtained in the course of the various treatments without heeding possible changes in the thickness of the object when computing the path difference.

Quality of double refraction

In dependence on the velocity of the extraordinary ray we speak of positive or negative double refraction. Native collagen fibres have a positive double refraction. In certain instances, in substances of different refractive indices,

collagen fibres may have a positive and a negative double refraction. (We do not refer to the phenol reaction which will be dealt with separately below.) A peculiar phenomenon is observed when using tanning agents (e. g., tannin) : at a refractive index of 1.4 to 1.5 the imbibition curve of the collagen becomes negative, to turn again positive around 1.7 (11). Double refraction of the fibres being thus influenced by the presence of tanning agents even in low concentrations, sections covered with substances of the said critical refractive indices and pretreated with a tanning agent, may bemisleading in estimating the quality of birefringence.

The phenol reaction

Ebner, in 1894, was the first to demonstrate that the positive double refraction of collagen fibres becomes negative upon the action of phenol. Since then, this is known to be a reaction specific to collagen fibres and tissue elements of mesenchymal origin. By observing the quantitative changes in the phenol reaction, Jobst [9] was able to register the very fine alterations occurring in the submicroscopic structure of connective-tissue fibres.

By thermal or chemical pretreatment it is possible to prevent the positive double refraction of fibres from turning negative upon the action of phenol. Metacollagen possesses this property (2, 3, 4, 21). (Fig. 3.) As this transformation of collagen fibres (metacollagenization) can be assumed to occur not only *in vitro* but also *in vivo*, due foresight must be exercised in evaluating the phenol reaction. There is a further phenomenon which merits to be pointed out. In our experiments, phenol dissolved in xylene (20 per cent) was used and it was found that on preventing the rate of concentration from changing with the evaporation of the xylene, the collagen fibres retained the negative double refraction they had acquired, while on allowing the rate of concentration to increase, the fibres contracted and the double refraction vanished.

Imbibition methods

In the order of increasing refractive indices, a great number of imbibition media has been listed in tables attached to publications on polarization microscopy. Only very few of these substances are suit for biological purposes, since none can be used that enter into chemical reaction with the object to be tested. The adverse consequences involved in a wrong choice of the imbibition fluid can best be demonstrated by the example of potassium mercuric iodide and glycerine, the two media recommended in almost every table. Diluted with water in various proportions, potassium mercuric iodide is apparently well suited to form an imbibition series, making it possible to use the same chemical

substance throughout and, by varying the rate of dilution, to obtain an imbibition fluid of any desired refractive index in the range from 1.33 to 1.73. On applying this substance to collagen fibres it was, however, found that at certain concentrations (20 to 40 per cent) the fibres began to contract, contraction was followed by relaxation, the fibres acquired a rubberlike elasticity, while double refraction decreased, to vanish altogether. Obviously, in this case the potassium mercuric iodide had entered into chemical reaction with the collagen fibres, and the fibres could of course no longer be regarded as native. Although it is thus an imbibition medium unsuitable for the study of connective-tissue fibres, beside potassium iodide, potassium mercuric iodide is widely used for other purposes, since it causes the fibres to contract already at room temperature (2, 3, 4, 20, 21, 22).

As regards the other medium, glycerine, this mixes intimately with water and, therefore, lends itself to the preparation of dilutions of refractive indices from 1.33 to 1.455. It is eminently suited for testing native collagen fibres, but not collagen fibres pretreated with heat or chemical substances since, apart from water, gelatin is soluble in glycerine, which with incipient gelatinization (metacollagenization) no longer functions as an imbibition medium but acts as a solvent.

Water used as an imbibition liquid sometimes plays a peculiar role. Of native collagen fibres it changes the double refraction in accordance with its refractive index in the imbibition series, but thermally or chemically pretreated fibres are markedly hydrophilic; they swell in water, their double refraction is reduced to zero, and then converted into a negative double refraction. As regards polarization optics, metacollagen arising upon the action of thermal or chemical pretreatment is very similar to gelatinous films. Placed in water, the gelatinous film swells intensely, is in isotropic phase, and if stretched, its double refraction, both qualitative and quantitative, depends on the tension to which it is exposed. If allowed to dry in stretched condition it retains its double refraction. Put back in water, it again contracts, swells, and then loses its double refraction. This phenomenon is not observed upon the action of any chemical substance (e. g., alcohols, oils, fat solvents) other than water and glycerine. By the analogy of gelatin, it can be supposed that like the stretched gelatinous film, the component of native collagen called metacollagen is in the anisotropic phase, and that it comes into isotropic phase after the total dissolution of the procollagen, resp. mucopolysaccharide, which process is accompanied by contraction-relaxation. Imbibition tests with native fibres showed that, in reference to the same fibre, the value at the lowest point of the curve of the path difference denotes the quantitative value of the double refraction of the metacollagen. The swelling due to the action of water alters the conditions of tension in the fibrils as well, and this is a point which must not be neglected when investigating into double refraction. Especially in elastic fibres is it clearly observable that with increasing the tension of the fibrils their double refraction increases. Fibres

stretched in water and thereafter dried display an intensive double refraction but when put back in water, they shrink, while their double refraction decreases. On the other hand, using alcohol, xylene, benzene, toluene, or some other lipophil substance, or oils, the fibres retain their double refraction.

For native fibres water, alcohol, xylene, toluene, and oils of high refractive indices can be employed to advantage as an imbibition series. For metacollagen fibres the series can be supplemented with 18 to 80 per cent, sugar solutions (nD = 1.36 to 1.49), since with sugar at these concentrations swelling is but limited.

A question concerning native collagen fibres

A great number of contributions have made it obvious that collagen fibres obtained with the most widely differing methods are all called native. Some authors apply this term to fibres dissolved in acetic acid, then dialysed, thereafter again precipitated ; others, to fibres which have been subjected to various fixing processes. Yet, in view of the most different organic and inorganic substances being capable of working their way into collagen fibres and, in doing so, altering their fundamental fibre properties, the term native should be applied with cautious restriction.

Parallel experiments were carried out with freshly prepared collagen fibres that were promptly used without fixation, as well as with fibres fixed in formaldehyde, embedded in paraffin, and then deparaffined. It was found that the fibres fixed in formaldehyde had lost that ability to contract in potassium iodide or potassium mercuric iodide, and which, as has been pointed out in our previous communications (2, 3, 4, 20, 21, 22), was involved in our fundamental experiments concerning the finest physicochemical properties, and those relative to the enzyme digestibility, of the fibres.

It is remarkable that as regards the phenol reaction, there is a difference between fibres fixed in formaldehyde and freshly prepared fibres promptly used without fixation. While the double refraction of both of them becomes negative, only the latter possess the capacity of contracting in high concentrations of phenol, contraction being followed by a gradual loss of their double refraction (Fig. 4).

The latest observations by means of the electron microscope likewise favour the view that the various substances which find their way into the fibrils change the periodicity of the latter. If, then, by the evidence of the electron microscope it is accepted that the fibre suffers a change in structure, there can be no reason for the assumption that from the point of view of polarization optics it remains intact.

Most of the fixing and embedding methods generally used as also temperatures above 37°C, involve the danger of irreversible changes in the finest structure.

On this consideration, our experiments and chemical reactions which were followed by measurements in polarized light, were mostly carried out promptly without applying chemical fixing agents. We found that fibres dried at a room temperature of 18°C were suitable for our experiments. Where it is not possible to utilize them promptly in the experiment or, and this is a more frequent occurrence, when a larger quantity of the same substance has to be stored, it is advisable to keep it in the frozen state. A freeze-drying apparatus is, of course, the means by which to secure ideal conditions for fixing.

With the phenomena described in the foregoing, investigators engaged in polarization microscopy are supposedly familiar, at least in part. Nevertheless, we believe our criticism of the imbibition methods and the description of our experience with native fibres and fixation, will serve useful purposes. Our principal aim in quoting the above examples has been to show that with connective-tissue fibres and other biological objects, the method to be applied in polarization microscopy is decided by the individual nature of the substance to be examined.

Summary

In the study of biological objects, among them connective-tissue fibres, the classical methods of polarization optics as applied in crystallography cannot be employed without certain restrictions.

In cross-textured collagen fibres the factors responsible for the intensity of double refraction can occasionally give rise to a state in which double refraction decreases. In such cases, preparing thinner sections will increase it.

Though thicker than native fibres, swollen fibres are less birefractive.

Upon the action of tanning agents (for instance, tannin), the imbibition curve of collagen fibres becomes negative.

Upon the action of phenol, the positive double refraction of stretched metacollagen does not become negative.

Of the imbibition media, potassium mercuric iodide and glycerine enter into chemical reaction with connective-tissue fibres.

In metacollagen fibres, water used as imbibition fluid fails to give rise to the change in double refraction which is observed in native collagen, as metacollagen fibres show no double refraction in water.

From the physicochemical point of view, collagen fibres should not be regarded as native unless freshly prepared and dried at 18° C or fixed in the frozen state. Fibres fixed in formaldehyde or pretreated with acetic acid lose their capacity to contract.

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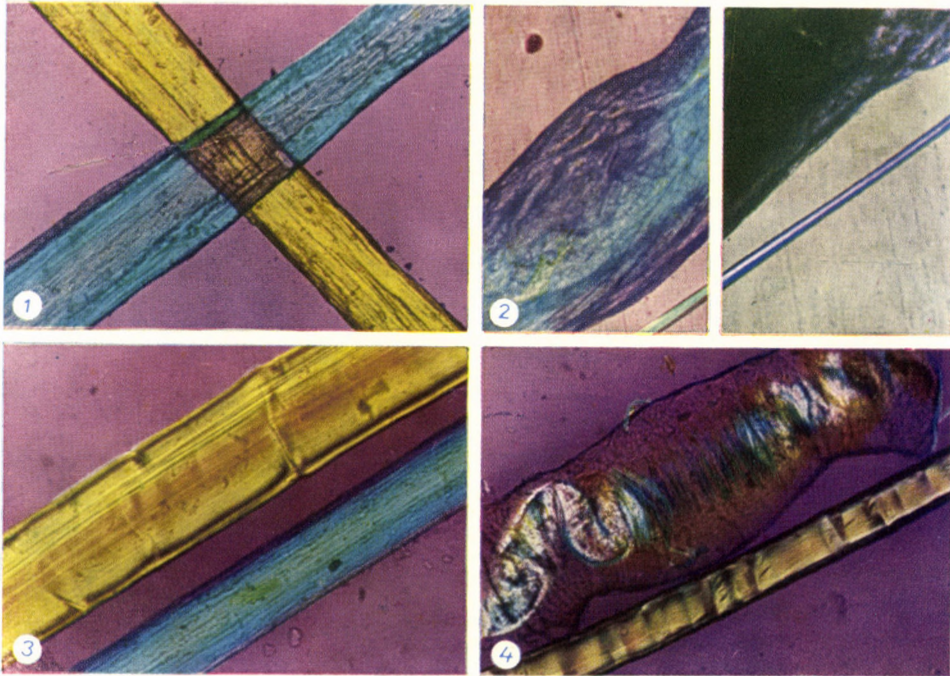

Fig. 1. Two collagen fibres of different double refraction. Crossings change the double refraction. (× 150)

Fig. 2. The thin fibres are native ; the thick ones, swollen in consequence of chemical pretreatment (ascorbic acid). On the left, it can be seen even through the gypsum plate that the double refraction of the thick fibre is decreased in relation to the thin one. On the right, Ehringshaus' quartz compensator [7] makes the difference in the double refraction of the native and swollen fibres still more conspicuous. (× 25)

Fig. 3. Phenol has converted the positive double refraction of the native collagen fibre into a negative one. This is visible on the yellow fibre. On the other hand, no change has been caused by phenol in the double refraction of the metacollagen (blue) fibre. (× 150)

Fig. 4. Following fixation in formaldehyde and embedding in paraffin, the positive double refraction of the thin yellow fibre has become negative upon the action of phenol. Without fixation, upon the action of phenol the collagen fibre swells, separates into two phases, ultimately to lose its double refraction. (× 150)

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ОСОБЫЕ ТОЧКИ ЗРЕНИЯ ПОЛЯРИЗАЦИОННО-ОПТИЧЕСКОГО ИССЛЕДОВАНИЯ СОЕДИНИТЕЛЬНОТКАННЫХ ВОЛОКОН

Д. САБО

При исследовании биологических объектов, в том числе и соединительнотканных волокон, классическими, применяемыми в кристаллографии методами можно пользоваться лишь при известных условиях.

Из факторов, определяющих степень двойного преломления, в случае перекрестного прохождения коллагенных волокон, может образоваться такое состояние, при котором двойное преломление уменьшается.

Набухающие волокна показывают — несмотря на то, что они более толстые, чем нативные волокна — уменьшение двойного преломления.

На действие дубильных веществ (напр. таннина) кривая пропитывания коллагенного волокна переходит в отрицательную.

На действие фенола положительное двойное преломление растянутого метаколлагена не становится отрицательным.

Из пропитывающих веществ раствор иодистой ртути калия и глицерин вступают в химическую реакцию с исследуемыми соединительнотканными волокнами.

Применяемая в качестве пропитывающей жидкости вода не вызывает в метаколлагенных волокнах наблюдаемое в случае нативного коллагена изменение двойного преломления, так как метаколлагенные волокна в воде не обладают двойным преломлением.

С физико-химической точки зрения только свежепрепарированные или сушеные при температуре в 18° С и фиксированные замораживанием коллагенные волокна можно рассматривать нативными. Фиксированные в формалине или обработанные уксусной кислотой волокна утрачивают способность к сокращению.

BESONDERE GESICHTSPUNKTE DER POLARISATIONSOPTISCHEN UNTERSUCHUNG VON BINDEGEWEBEFASERN

D. SZABÓ

Bei der Untersuchung biologischer Objekte, so auch der Bindegewebefasern, können die klassischen, in der Kristallographie angewandten Methoden der Polarisationsoptik nur mit gewissen Einschränkungen angewandt werden.

Die den Grad der Doppelbrechung bestimmenden Faktore können bei Kollagenfasern mit gekreuztem Verlauf einen Zustand hervorrufen, bei welchem sich die Doppelbrechnung vermindert. Werden in solchen Fällen dünnere Schnitte hergestellt, wird die Doppelbrechnung stärker.

Gequollene Fasern zeigen selbst wenn sie dicker sind als die nativen Fasern, eine Verminderung der Doppelbrechung.

Auf Wirkung von Gerbstoffen (z. B. Tannin) geht die Imbibitionskurve der Kollagenfasern ins Negative über.

Auf Wirkung von Phenol wird die positive Doppelbrechung des gedehnten Metakollagens nicht negativ.

Unter den Imbibitionsstoffen treten Kaliummerkurijodid und Glyzerin mit den Bindegewebefasern in chemische Reaktion.

Das als Imbibitionsflüssigkeit angewandte Wasser verursacht in den Metakollagenfasern nicht die bei nativem Kollagen beobachtete Veränderung der Doppelbrechung, da die Metakollagenfasern in Wasser keine Doppelbrechung aufweisen.

Vom physikalisch-chemischem Gesichtspunkt können nur frisch präparierte, bzw. bei 18°C getrocknete, sowie mit Gefrieren fixierte Kollagenfasern als native Fasern betrachtet werden. In Formalin fixierte, oder mit Essigsäure vorbehandelte Fasern verlieren ihre Kontraktionsfähigkeit.

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