THE DOUBLE REFRACTION OF CHITIN

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I

Chitin, a nitrogen-containing polysaccharide, is the chief structural element in the cell walls of fungi and in the integument of arthropods. Structurally chitin resembles cellulose, the unit of structure being the acetyl-glucosamine residue instead of the glucose residue (Meyer and Mark, 1930). Organized chitin is optically anisotropic and exhibits birefringence which is ordinarily positive[1] in relation to the principal axis of the structure in which it occurs. Attempts to analyze the double refraction of chitin by means of the imbibition technique of Ambronn (Ambronn and Frey, 1926) have ascribed to it two components: (1) a positive double refraction due to the parallel arrangement of minute, submicroscopic micelles[2] of chitin imbedded in another medium (*form birefringence*), and (2) a negative double refraction of the crystalline micelles themselves. The latter, if correct, is remarkable since the micelle of cellulose is strongly optically positive.

The theory of the imbibition technique as developed by Ambronn may be briefly stated as follows (see Ambronn and Frey, 1926; Schmidt, 1934; Frey-Wyssling, 1935). Bodies exhibiting form birefringence are two phase systems, consisting usually of a solid framework permeated by a continuous medium such as gas or liquid. If the dimensions of the solid units and the spaces between them are small in

[1] Birefringence is referred to as positive in relation to some axis when the greatest index of refraction of the substance, n_γ, is parallel to, negative when n_γ is perpendicular to that axis.

[2] Micelle is here used in the sense of Naegeli to denote an aggregate of submicroscopic dimensions of smaller chemical units. Polarization optics can assign no definite limits of size to the micelle, but can only tell us that it is an elongate structure. For cellulose, a recent estimate of the micellar dimensions is 60 × 60 × 750 Å (Frey-Wyssling, 1935).

comparison with the wavelength of light, the material will be birefringent provided (1) the solid units are anisodiametric and oriented parallel to one another, and (2) the continuous medium has a different index of refraction from the solid particles.

The commonest case of form birefringence is that where the solid particles have the form of little rods oriented parallel to their long axis. For this case the following equation has been derived:

$$n_a^2 - n_o^2 = \frac{\delta_1\delta_2(n_1^2 - n_2^2)^2}{(\delta_1 + 1)n_2^2 + \delta_1 n_1^2}$$

where

n_a = index of refraction of the extraordinary ray (in the system as a whole)
n_o = index of refraction of the ordinary ray (in the system as a whole)
n_1 = index of refraction of the solid component
n_2 = index of refraction of the continuous medium
δ_1, δ_2 = relative volumes of the two components $(\delta_1 + \delta_2 = 1)$.

The strength of double refraction is given by the difference $n_a - n_o$, and it is clear that this will be determined by the difference in the refractive indices of the two phases, $n_1 - n_2$. If n_2 is made equal to n_1, double refraction of the form type disappears and the system as a whole is isotropic. Ambronn's imbibition technique consists in soaking the material under investigation in a series of penetrating fluids having different values of n_2, and measuring the resulting birefringence. By this means a U-shaped curve is obtained when double refraction is plotted against n_2, having a minimum where $n_2 = n_1$.

If the solid phase consists of particles which are themselves crystalline and anisotropic, the minimum of the imbibition curve should not be at zero birefringence, but should have a positive or negative value due to the solid phase alone. From the course of the imbibition curve, then, it should be possible to determine to what extent the general birefringence is due to (1) an oriented arrangement of particles, and (2) crystallinity of the particles themselves. This paper deals with the interpretation and evaluation of such imbibition curves for chitin.

II

Following a study of the birefringence of fungus chitin, a source of thicker chitin was sought for and found in the marginal hairs of the

crayfish's carapace. A variety of evidence points to the chemical similarity of chitin from plant and animal sources (Rammelberg, 1931; Khouvine, 1932; Zechmeister and Toth, 1934). All the measurements described below were made on crayfish chitin.

A small piece of the edge of the crayfish's carapace was cut off, with marginal hairs attached. These hairs are attached to and continuous with the chitinous inner layer of the shell which, in the case of the lobster, was used by Möhring (1926) as a source of chitin. After boiling in saturated KOH following the method of Campbell (1929), the hairs acquire a violet color with iodine in KI plus dilute H_2SO_4, dissolve in 70 per cent H_2SO_4, and are therefore judged to be chitin. Before imbibition, fresh hairs were boiled briefly in 5 per cent HCl to remove any calcareous incrustations, and then in 10 per cent KOH to dissolve any other materials in the hair which might impede penetration. This procedure was found to facilitate imbibition greatly without sensibly altering the double refraction.

Retardations were measured by means of a Berek compensator used in a Fuess polarizing microscope. Blue light of high intensity was used throughout, obtained by focusing the beam of a 250 watt projection lamp on the substage mirror, and screening out heat and other portions of the spectrum with a 2 cm. filter of saturated aqueous $CuSO_4$. Wavelength 486 mμ was used in all the computations.

Imbibition was carried out in hollow-ground glass slides containing about 0.2 cc. of fluid and closed by a large cover-glass. A small air bubble was enclosed, which, when the slide was tilted back and forth, served to give excellent mixing of the fluid in the chamber. Being sealed by capillary force between the slide and cover-glass, the chamber could be heated to 60°C. without serious evaporation of one constituent of a mixture. With heating, imbibition occurred very rapidly, usually no further change being noted after 5 minutes. In doubtful cases an imbibition time of several days was allowed.

The refractive indices of all liquids and mixtures below $n = 1.60$ were measured with a Pulfrich refractometer, and the values are given below as n_D at room temperature (approximately 25°C.). Higher values of n were obtained by extrapolation of mixture curves measured over the working range of the instrument. All retardation values are calculated from averages of at least three pairs of settings of the compensator.

Three principal series of imbibition fluids were used: (1) water-mercuric KI mixtures,[3] (2) xylol-methylene iodide mixtures, and (3) ethyl alcohol (or xylol)-iodobenzene mixtures. The results are given in Figs. 1, 2, and 3. In addition,

[3] A saturated solution of mercuric potassium iodide (potassium iodomercurate, Thoulet solution) was prepared by adding KI and HgI_2 in excess to water at room temperature. This saturated solution has a refractive index of approximately 1.73.

a short series was run with water-glycerol mixtures (Fig. 4). All of these measurements were made at one particular spot on the same crayfish hair within a period of 2 weeks. As may be seen from duplicate measurements, the curves were reversible and reproducible.

Sources of error other than instrumental in this kind of work are chiefly due to (1) incomplete penetration of the fluids, (2) swelling or shrinkage of the material with possible alteration of the shape and relative volumes of the micelles and the spaces between them, (3) chemical effects of the fluids on the imbibing material. With crayfish chitin penetration was taken to be complete when further heating and rinsing in fresh fluid produced no additional change in birefringence. Measurable swelling did not occur even in the mercuric KI solutions. The possible occurrence of unforseen chemical or adsorption effects is a real one. Cinnamic aldehyde, benzaldehyde, and to some extent aniline were found to reverse the normal positive double refraction of chitin, producing negative double refraction. This type of action was studied with collagen fibers by von Ebner (1894), and attributed by him to an oriented adsorption of imbibed molecules on the colloidal framework.

III

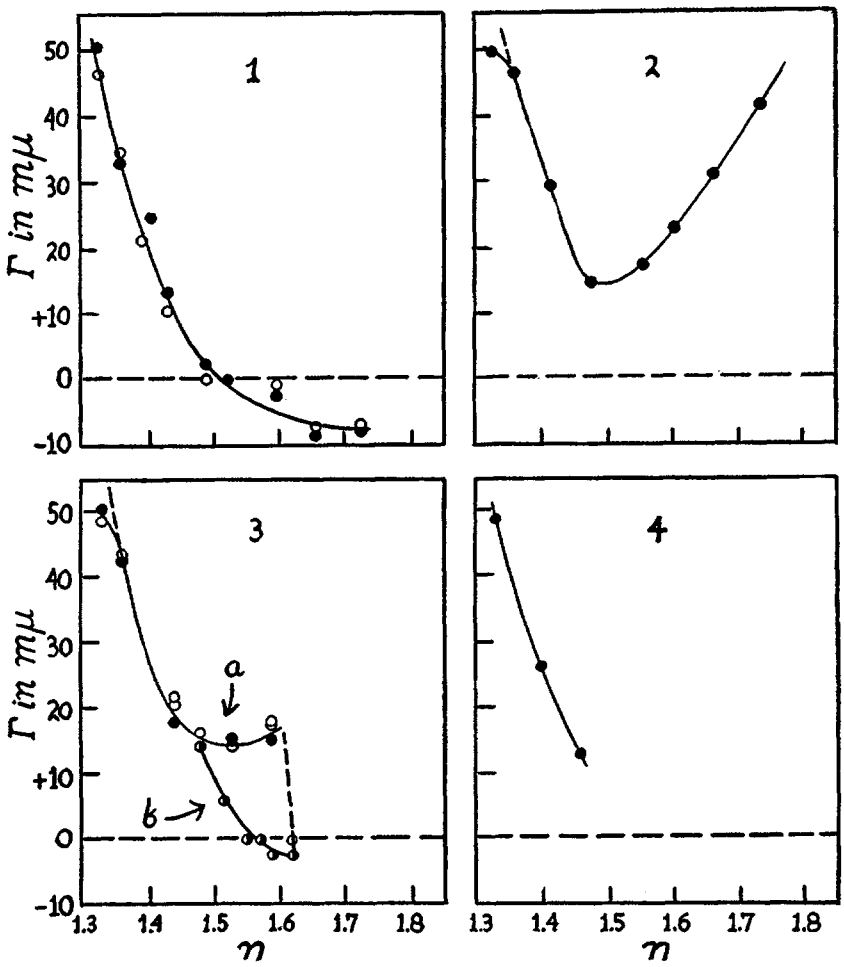
The commonly accepted view of the fine structure of chitin is due to Möhring (1926), who worked with the decalcified inner layer of the lobster carapace. Using mixtures of water and mercuric KI, he obtained a U-shaped imbibition curve which twice cut the line of isotropy, passing through a minimum with a residual value of *negative* birefringence when imbibed with fluid of refractive index 1.61. In saturated mercuric KI at $n = 1.73$ birefringence is again positive. The interpretation made of this curve is that positive form birefringence is abolished at $n = 1.61$, and the residual negative birefringence is due to the optically negative crystallinity of the chitin micelle. Schmidt (1934) has come to similar conclusions from qualitative observations on the treatment of chitinous insect's tendons with mercuric KI solutions. X-ray studies (Meyer and Mark, 1930) show that chitin is partially crystalline.

Whether or not Möhring's interpretation is correct in theory, its reasonableness depends on the U-shaped character of the imbibition

curve, which twice cuts the line of isotropy. Careful repetition of the mercuric KI imbibition of crayfish chitin yielded a curve (Fig. 1) the left-hand limb of which resembles Möhring's, but with no trace of a secondary rise at the highest values of n. The reproducibility of the curve is shown by two series of measurements, taken a week apart, plotted in Fig. 1. The fact that the curve approaches a limiting value of negative double refraction with the highest concentrations of mercuric KI suggests a progressive, oriented association of ions or molecules of the solution with the chitin micelles, rather than a purely physical effect based on increase of refractive index of the intermicellar fluid.

Imbibition with xylol-methylene iodide mixtures gives a curve covering the same wide range, but differing radically from that obtained with mercuric KI solutions. Here the material is passed from water into absolute ethyl alcohol, then into xylol, and then into mixtures of xylol with methylene iodide. The left-hand limb of the curve (Fig. 2) runs parallel to but somewhat higher than the previous one (Fig. 1), and there is evidence that this net increase in double refraction is due to dehydration in the passage from water into alcohol. The remarkable aspect of this curve, however, is that with progressive addition of methylene iodide to xylol it rises sharply to high values of positive double refraction. This U-shaped curve, then, could be interpreted to mean that in addition to positive form double refraction chitin possesses a residual positive double refraction of the micelle, and that the crystalline micelle is optically positive.

It is conceivable that the marked difference between the curve for mercuric KI and for xylol-methylene iodide mixtures might be due to the fact that in one case the chitin micelle was hydrated, in the other dehydrated. To test this idea, mixtures of another non-aqueous liquid, iodobenzene, were made up with (1) absolute ethyl alcohol, and (2) with xylol. The results of imbibing the same piece of chitin with these fluids are shown in Fig. 3. The xylol-iodobenzene curve continues to fall steeply beyond the point for pure xylol, cuts the line of isotropy at $n = 1.55$ to 1.57, and gives a very weak negative double refraction in pure iodobenzene ($n = 1.62$). The alcohol-iodobenzene curve is anomalous. It reaches a plateau or a slight minimum at $n = 1.53$, and keeps approximately that value in mixtures containing



Imbibition curves of crayfish chitin. Abscissa: refractive index of imbibing liquid; ordinate: retardation, Γ, in mμ for blue light. Dotted lines represent isotropy (Γ = 0).

FIG. 1. Mercuric KI imbibition. The top pair of points is for water, all others for water-mercuric KI mixtures. Solid and open circles are separate series.

FIG. 2. Points on the left are, reading from the top, for water, ethyl alcohol, alcohol-xylol mixture, xylol. The right-hand limb of the curve is for xylol-methylene iodide mixtures, ending with pure CH_2I_2 (n = 1.74).

FIG. 3. The top pairs of points are for water, then ethyl alcohol. Curve *a*, alcohol-iodobenzene mixtures (open and solid circles are separate series); curve *b*, xylol-iodobenzene mixtures (half-solid circles).

FIG. 4. Imbibition with water, water-glycerol mixture, glycerol.

more and more iodobenzene. In pure iodobenzene, however, it falls suddenly to isotropy or slight negative double refraction. This discontinuity may be ascribed to either incomplete penetration or to selective binding of a constituent of the mixture. In any case, the iodobenzene imbibition series show that isotropy and negative double refraction can be obtained with non-aqueous fluids as well as with aqueous mercuric KI solutions, so the presence or absence of water cannot be the reason for the different types of curve.

Imbibition with water-glycerol mixtures gives the fragmentary curve in Fig. 4, which runs approximately parallel to and in between the imbibition curves previously described. Unfortunately the refractive index of pure glycerine is so low, $n = 1.46$, that the course of the curve at the higher, critical values of n cannot be judged.

IV

Which set of data are we to believe represents a normal imbibition curve of the Ambronn type? According to the simplest form of the theory, double refraction due to the parallel arrangement of rod-like submicroscopic elements imbedded in a continuous medium of different refractive index is abolished if the continuous phase has the same refractive index as the dispersed phase. The imbibing fluid is assumed to act only by virtue of its refractive index, and must therefore enter into no chemical reactions or oriented adsorptions with the dispersed phase. Relatively inert structures, such as the silicious shells of diatoms or the silicious skeleton of the barley awn (Frey, 1926) exhibit positive form double refraction, and their imbibition curves are of the simple U-shaped type demanded by the theory. More complex organized structures, such as the polysaccharides, are well known to enter into a variety of adsorption compounds, and von Ebner (1894) long ago described the association of certain phenols and aromatic aldehydes with collagen, reversing the sign of its double refraction. Chitin also forms such associations, especially with aldehydes. In interpreting the results of imbibition curves of such substances, therefore, great care is necessary, particularly since the choice of fluids having refractive indices above 1.5 is limited to a few.

All of the curves agree in showing a rapid decrease in the double refraction of chitin between $n = 1.33$ and $n = 1.49$. The point for

xylol ($n = 1.49$), a hydrocarbon, seems particularly reliable. There can be little doubt, therefore, but that a very large proportion of the positive birefringence of chitin is due to the form element, vastly more than in the case of cellulose.

The further course of the curve depends wholly on what imbibing fluids are used. The methylene iodide curve (Fig. 2) is pleasing if one hopes to obtain a U-shaped curve, and it may be significant that it can be drawn to inflect near $n = 1.525$ which Becking and Chamberlin (1925) found to be the refractive index of crab and insect chitin. The course of the alcohol-iodobenzene curve (Fig. 3) appears to have a minimum in the same region. Pure iodobenzene, however, produces feeble negative double refraction, and an aqueous mercuric KI mixture of $n = 1.525$ produces isotropy. Since there is no certain way of disentangling the influence of changing refractive index from chemical or adsorption effects, it is impossible at present to favor one of these procedures over another. The complex ions present in the mercuric KI solutions would appear, however, to make use of this reagent particularly unsuitable. The course of the mercuric KI imbibition curve certainly points to an action on chitin beyond that expected merely on the basis of its refractive index. It is unsafe, then, to draw conclusions from such experiments about the crystalline nature of the chitin micelle.

SUMMARY

The double refraction of the chitinous hair of the crayfish is positive with respect to the axis of the hair, and is largely caused by the arrangement of submicroscopic, elongated chitin particles parallel to this axis (*form birefringence*). Using a series of relatively unreactive liquids and fluid mixtures which permeate the chitin framework, the type of curve relating double refraction and refractive index of the imbibed fluid is found to depend greatly on the chemical nature of the fluid. Either a positive or a negative residual birefringence may be found, depending on the choice of imbibing liquid. Separation of form and crystalline elements in double refraction by means of Ambronn's imbibition technique is therefore unsafe in a system like chitin, where some type of oriented association of the imbibed molecules with the chitin framework is prevalent.

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