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INTRODUCTION

An ordinary biological microscope can only be used to examine such objects which tend to vary in their light absorption versus medium that surrounds them thus showing a certain amount of natural contrast. These objects are often called amplitude objects since they result in a change of amplitude in the light being transmitted. However, the nature knows also such microobjects and structures which produce no variations in the light being absorbed and differ from the surrounding medium merely by their refractive index or thickness. These use to be called phase objects since they cause only a shift in the phase of light wave being transmitted. In a simple bench microscope, they are hardly visible or entirely invisible in the transmitted light as human eye remains insensitive to changes in the light wave phase and reacts only to variations of luminous intensity i.e. variations of the light wave amplitude (the luminous intensity being proportional to the square of amplitude).

The phase objects must be previously stained to be distinguishable by means of an ordinary biological microscope. But, the staining of specimens appears to be rather troublesome and involves a variety of undesirable consequences. In particular, the living cells and tissues are not suitable for staining as staining causes their death. Hence, whenever a normal biological microscope is being used the examination of perfectly transparent living microorganisms, which do not absorb light at all, is greatly limited.

At present, these are the phase contrast microscopes which have found almost universal use in observation of phase objects. The disadvantage of those microscopes, in addition to such drawbacks as e.g. the halo effect, low contrast in the overlapping densely arranged structures, and poor representation of extended objects relatively large in their transverse dimensions, consists in the fact that their applications have been in principle reduced to qualitative and descriptive investigations only.

The introduction of phase contrast has made it possible to assess quickly whether the refractive index of an object under examination is smaller or greater than of the surrounding medium. In certain cases it permits even an accurate assessment of such a refractive index by selection of an appropriate immersion liquid, but in principle there exist no further possibilities of quantitative investigations to be made by the

phase contrast method without taking into account the potentiality of measuring linear and two-dimensional magnitudes.

Much greater potentialities to carry out the quantitative investigations are being offered by the interference microscopy in general, and the BIOLAR PI polarizing interference microscope or a UPI polarizing interference attachment with a BIOLAR biological microscope in particular.

Polarizing interference microscopes are being used to carry on observations of various microobjects which either produce a shift in the phase (transparent) or in the amplitude (light absorbing ones) of light wave being transmitted. They also serve the purpose of measuring optical path difference (phase shift), gradient of the optical path difference thickness, refractive index, birefringence, tangential angle, microsurface irregularities, concentration of substances, content of dry mass in the cells, light transmittance and other physical quantities.

A BIOLAR PI Microscope is suitable for both qualitative and quantitative investigations being effected by the following interference methods: fringe method, differential method and uniform field method with a high image shearing effect.

The polarizing interference microscopes find numerous uses primarily in the biological and medical sciences (especially in cytology, histology, morphology, biochemistry and microbiology), being also very useful in the physical chemistry, crystallography, mineralogy, textile manufacture, in the production of thin-layer circuits and in other fields of science and technology.

CHAPTER 1

1. PRINCIPLE OF OPERATION FOR A POLARIZING INTERFERENCE MICROSCOPE INCLUDING ONE BIREFRINGENT PRISM

For optical system diagram and general principle of operation of a polarizing interference microscope refer to Fig. 1. The polarizing interference microscope differs from a conventional biological microscope basically in that it incorporates a polarizing interference system consisting of a birefringent prism W_1, polarizer P, analyser A and a slit diaphragm D or an appropriate quartz compensator.

The birefringent prism W_1 represents a special kind of the Wollaston prism. Located just after objective Ob it can move both in the parallel as well as perpendicular directions versus the microscope centre line. Function of this prism consists basically in the splitting (bifurcating) of incident light beam into two beams viz. an ordinary and an extraordinary beam, and in producing an adequate phase shift between light beams so split. The angular bifurcation Σ of the rays of light increases along with the growing angle of refraction φ of the birefringent prism.

The polarizer P and analyser A are intended for the linear polarization of light. They are, in fact, ordinary standard polarizing filters, the so-called polaroids, which rotate about an axis being parallel to the path of light in the microscope. Polarizer P is accommodated in the microscope illuminator part underlying the condenser whereas analyser A remains between the birefringent prism and an eyepiece.

System composed of the polarizer P and analyser A, with a birefringent prism W_1 located in between, produces in the transmitted light a number of rectilinear interference fringes whose intensity reaches a maximum when the planes of polarization for both polarizer P and analyser A become mutually perpendicular or parallel and form an angle of 45° versus the refracting edge of birefringent prism W_1 (this edge adjacent to angle φ is perpendicular to the plane of Fig. 1). Fringes thus produced appear inside the prism W_1 or at a certain distance under-

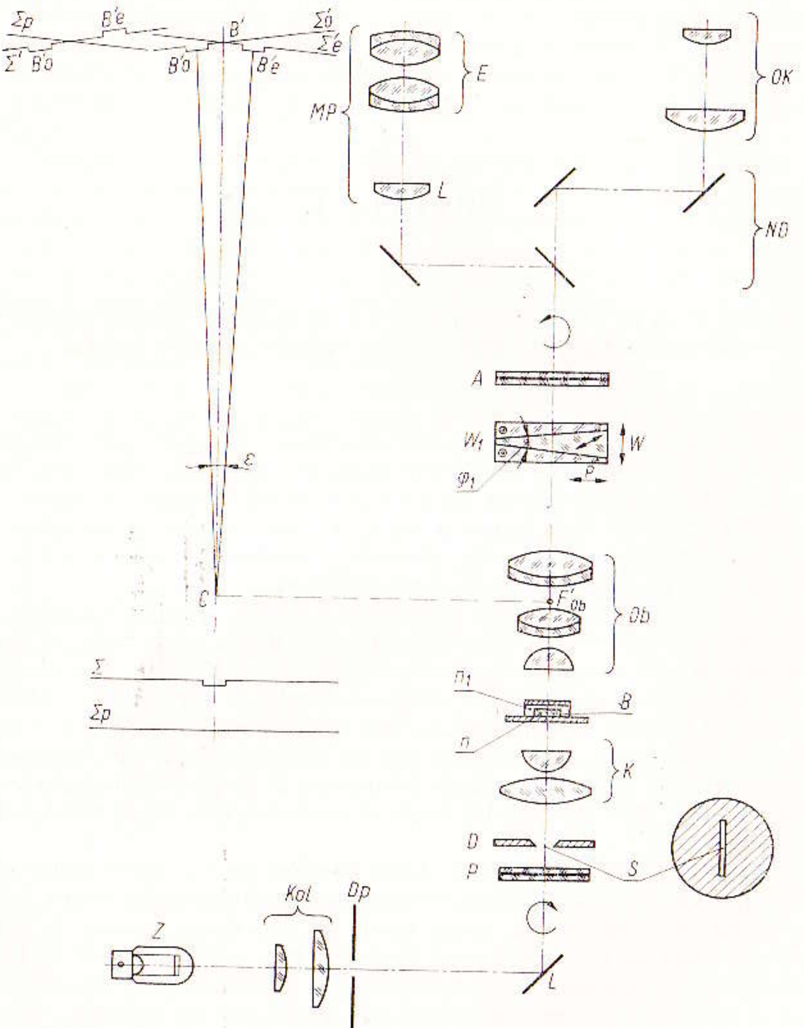

Fig. 1. Diagram illustrating optical system of a polarizing interference microscope with only one birefringent prism

Z — Source of light; *Kol* — Illuminator collector; *DP* — Field diaphragm; *P* — Polarizer; *D* — Slit diaphragm; *S* — Slit; *K* — Condenser; *B* — Specimen; *Ob* — Objective; W_1 — Birefringent prism; *A* — Analyser; *ND* — Binocular body; *Ok* — Eyepiece; *MP* — Auxiliary microscope; *E* — Eyepiece; *L* — Objective (for explanations regarding other symbols see text)

neath at points *C* where bifurcation of light rays usually takes place. The interfringe spacing h remains constant throughout the entire lenght of the prism and can be expressed as follows:

$$h = \frac{\lambda}{\varepsilon} \tag{1}$$

where λ — light wavelength.

Slit diaphragm *D* has been located in the focus of condenser *K*, the slit *S* being parallel to the refracting edge of birefringent prism W_1. Slit *S* and the condenser *K* form a collimator out of which beams of coherent light are delivered susceptible to interference in the microscopic image plane. Interference fringes in this plane can be produced only if slit *S* has been adequately narrowed whereas interference fringes in the exit pupil of objective *Ob*, referred to above, can be observed no matter what the width of slit *S* is.

Let us assume that the phase object *B* (Fig. 1) under examination has the form of, say, a narrow strip having thickness t. This object is isotropic, perfectly transparent, and its refractive index n differs from that of the surrounding medium n_1 (e.g. $n > n_1$). Under such circumstances, the flat light wave Σ_p coming out of the condenser *K*, after a linear polarization in the polarizer *P*, will be subject to a phase lag within the space occupied by object *B* thus taking on the form of wave Σ. This wave enters objective *Ob* where it becomes split by the birefringent prism W_1 into two waves, an ordinary and an extraordinary wave, polarized versus each other in perpendicular planes. The analyser *A* transmits only these wave components which are parallel to its own direction of light oscillation.

With the light rays allowed to pass through the analyser *A* two waves are produced, an ordinary wave Σ'_o and an extraordinary wave Σ'_e, linearly polarized in the planes at right angles versus each other. These waves can interfere so that after their superposition in the image plane an interference is generally obtained in the form of rectilinear interference fringes liable to deformation in the opposite directions where the ordinary, B'_o, and extraordinary, B'_e, images of object *B* (Fig. 2) are usually produced.

Interference image appearing in the microscope image plane can be interpreted as a superposition of flat waves Σ_p and Σ', which are liable to a phase displacement being advanced within the image area of object under examination and delayed within the second image area respectively. This is like the observation in an ordinary interferometer

of two objects having $t(n_1-n)$ and $-t(n_1-n)$ optical thicknesses and separated from one another by a value r to be found from the formula:

$$r=\frac{l\cdot\varepsilon}{G} \qquad (2)$$

where:

l — distance between the point of bifurcation of light rays and the microscopic image plane ($l=CB'$);

ε — angular bifurcation in radians;

G — objective magnification.

Thus obtained interference image resembles this of the generally known interference patterns equal in their thickness and coming to be seen on an air wedge. The only difference consists in two sheared images being obtained for one and the same object, with interference fringes deviated in such images in the opposite directions. A situation is created advantageous in many cases since by measurement of a double deviation of fringes $2d$ (Fig. 2) a better accuracy of measurement is possible to be achieved for the optical path difference.

When the monochromatic light is applied, the interference image consists of alternate dark and bright fringes. Whenever the polarizer P and analyser A are crossed, dark fringes are formed in those places where optical path difference between the interfering waves Σ'_o and

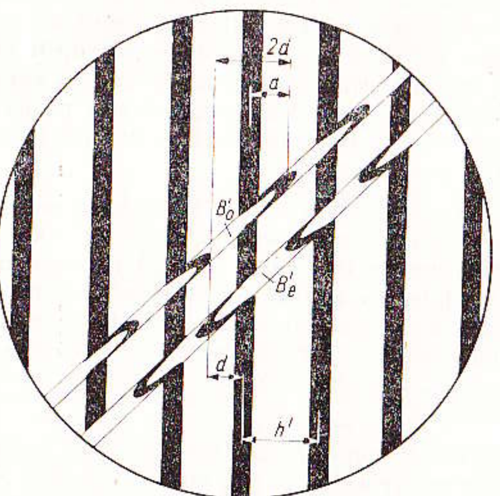

Fig. 2: Image of a narrow strip in an interference fringed field
B'_o — Ordinary image; B'_e — Extraordinary image; h' — Inter-fringe spacing; d — Deviation of interference fringes

Σ'_e equals zero or a whole multiple of the wavelenght λ of light having been used. However, if the polarizer and analyser are set in parallel, dark fringes appear at points where the optical path difference between those interfering waves is equal to an odd multiple of $\frac{\lambda}{2}$. When using white light, interference fringes — except for the zero fringe at crossed polarizer and analyser — are coloured whereby their intensity becomes lower and lower with the increase in interference order to finally fade away completely (Fig. 45).

The method described herein, and hereinafter called „fringe method", appears to be particularly suitable in the examination of oblong and minor separated objects whose images can be split by half at least.

Such oblong objects include fibres, thin strips or edges and should be preferably set up so as to be at 45° versus the direction of interference fringes (Fig. 2) approximately.

By measuring the inter-fringe spacing h' and deviation of fringes d, and by knowing refractive index n_1 of the surrounding medium, one can determine refractive index n of the object under examination if only he knows its thickness t, or evaluate the thickness t if the refractive index n is known. When applying two immersion liquids of a known refractive index n_1 and n_2 respectively, it is possible to determine t and n at a time. This will be discussed in detail in Chapter 6.

The interference fringe image can be obtained in the microscope viewing plane only when the plane of interference fringes for the birefringent prism W_1 does not coincide with the focus F'_{ob} of the microscope objective Ob. When the birefringent prism is brought up nearer to the objective Ob, then the birefringent fringes do become increasingly extended until they change into wide bands so as to finally cover the entire field homogeneous in its appearance. Such a situation occurs when the point of light rays being split fully coincides with the viewing focus of the F'_{ob} objective. Then, the Σ'_o and Σ'_e wave fronts neither intersect nor are inclined versus each other, like in the fringe method described hereinabove, but they are mutually parallel (Fig. 3). The optical path difference Ψ does not change continually but remains constant now, except for the space where split images B'_o and B'_e of the object B are produced. As a consequence, background of the microscopic field of view comes to be seen as being uniform in its colour whereas the object under examination is observed as a change in said colour at the point where the split B'_o

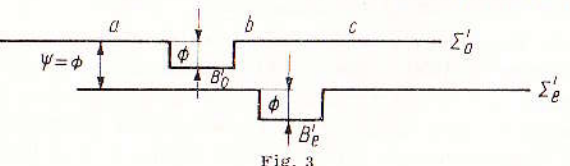

Fig. 3

and B'_e are being formed, or, as a change in the level of brightness where monochromatic light finds use. Field brightness depends in this case on the optical path difference Ψ between waves Σ'_o and Σ'_e. With the crossed polarizer and analyser, darkness of the field background will reach its maximum if the optical path difference Ψ is zero or a whole multiple of the applied light wavelength λ. For optical path difference Ψ equal to an odd multiple of $\lambda/2$ the field of view will remain, on the other hand, at its maximum bringhtness. Where intermediate values are involved, the level of field bringhtness will vary accordingly. With the polarizer and analyser set in parallel versus each other, the situation will be reversed, i.e. the minimum darkness of the field of view will be for $\Psi = \lambda(m = 0, 1, 2...)$ and the maximum one for

$$\Psi = m\frac{\lambda}{2}(m=1, 3, 5.).$$

When the white light is being used, it will be generally the colour that shall change along with the varying optical path difference instead of brightness. For interference colours depending on the optical path difference Ψ variations, both at crossed and parallel polarizer and analyser, refer to Table I. And so e.g. a dark colour will be produced at $\Psi=0$ with the crossed polarizer and analyser; this colour will change with the growing Ψ into grey, white, and various hues of the yellow and red colour. At $\Psi=565$ nm, the so-called sensitive colour of the primary interference order comes to be seen. This is a purple colour characteristic in that it easily changes into red or violet if only the optical path difference Ψ undergoes the slightest alteration. Accordingly, a very high leap in colour will be usual for every change in the optical path difference. With the parallelly set polarizer and analyser, the sensitive colour of the primary interference order will be produced at $\Psi=280$ nm.

The value of optical path difference Ψ between the interfering waves Σ'_o and Σ'_e will depend on the point at which the beam of light will pass through the birefringent prism W_1 (Fig. 1). By shifting this prism at right angles to the microscope centre line (this direction is shown by the arrow p in Fig. 1), the operator is in a position to change the optical path difference Ψ in a continuous manner thus controlling also the level of brightness or darkness of the microscopic field and this of the image being viewed. This provides means for an adequate selection of viewing conditions under coloured interference contrast and renders possible measuring of the optical path difference Φ caused by the object under examination.

To explain this feature in a more detailed manner let us assume that the observation takes place in the white light conditions, with the birefringent prism set to the primary-order sensitive colour (i.e. with the polarizer and analyser in their crossed positions). The background of the microscopic field of view (space a, b, c in Fig. 3) will

then become purple. On the other hand, in these sections of the field of view where the ordinary image B'_o and extraordinary image B'_e of the object under examination are being formed the colour will be different since at B'_o the optical path difference Ψ between interfering waves Σ'_o and Σ'_e will be smaller and at B'_e greater by the value of optical path difference Φ caused by the object under observation (B). Accordingly, image B'_o will note optical path difference $\Psi=(560-\Phi)$ nm and image B'_e $\Psi=(560+\Phi)$ nm.

For instance, assume $\Phi=100$ nm to find that optical path difference Ψ at B'_o and B'_e will be 460 nm and 660 nm respectively.

As presented in Interference Colour Table, image B'_o will be orange and B'_e blue in their hue. For $\Psi=0$ (with both polaroids crossed), i.e. for birefringent prism set to dark colouration, both images will be identically lavender-grey in their tinge. With the birefringent prism set to another field colouration, the split images will of course take another colour too. Therefore, by setting the birefringent prism to different colourations of the field of view and by assessing change taking place in the split images of the object under observation one can easily find optical path difference Φ for such an object in respect to the surrounding medium.

This method, though being quite satisfactory in numerous applications and leading to good results, is nevertheless highly subjective. While considering this fact, a more objective method for measuring the optical path difference — also straightforward in use and ensuring good accuracy of measurement — has been introduced in the polarizing interference microscopy. This consists in producing first an initial (standard) colouration of the field background in the ordinary or extraordinary image to carry out consequently measurement of the transversal shift of the birefringent prism performed during this operation.

To achieve this, it will be preferable to make use of the dark zero-intereference-order colour (with the crossed polarizer and analyser). More details about this step find in Fig. 4. The magnitude to be

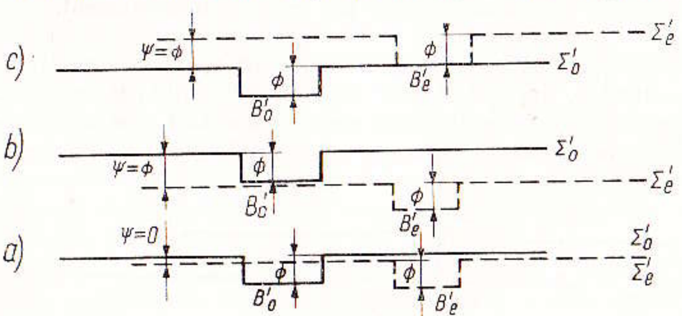

Fig. 4

measured in this case is the optical path difference Φ caused by the object under observation. Assume now that the birefringent prism has been set in the first place to the dark background of the viewing field (Fig. 4a), and that the birefringent prism position is p_0 (this is like zero position). While moving the birefringent prism transversely from this position, in either direction, wave surfaces will separate due to phase displacement so that either ordinary wave Σ'_o will be in advance of the extraordinary wave Σ'_e or the extraordinary wave Σ'_e will precede the ordinary one Σ'_o. As soon as the optical path difference Ψ between waves Σ'_o and Σ'_e reaches outside spaces B'_o and B'_e, the value of the optical path difference Φ caused by the object under examination, then either the ordinary image B'_o (Fig. 4b) or the extraordinary one B'_e (Fig. 4c) will receive a dark colouration, i.e. the former colouration of the viewing field background. Let us now assume that the maximum darkening of the ordinary or extraordinary image corresponds to position p_1 of the birefringent prism. In these circumstances, the optical path difference Φ will be:

$$\Phi=(p_1-p_0)\Psi_p \tag{3}$$

where: Ψ_p is the optical path difference between waves Σ'_o and Σ'_e per unit of the birefringent prism transversal shift.

It can be easily proved that

$$\Psi_p=\frac{\lambda}{h} \tag{4}$$

By reading prism positions p_1 and p_2, in which first one and then the second image of the object being observed become maximally darkened, one can find the double value of:

$$2\Phi=(p_2-p_1)\Psi_p \tag{5}$$

contributing to an increase in the accuracy of measurement.

Where objects of a varying thickness or varying refractive index are investigated, the optical path difference Ψ within the space of doubled images remains a variable and not constant (Fig. 5). Therefore, also colour or brightness will change accordingly at various points of

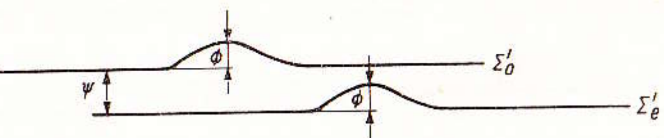

Fig. 5

the image. Considering a change in colour it will be possible to estimate variations of the optical path difference within the object, or by darkening particular sections of the image to determine optical path difference at any point of the object under examination.

So far, the case of a total splitting of both the ordinary and extraordinary images of the object under observation has been discussed. The conditions of observation, as referred to above look, however, a little different if the images remain not entirely separated but partially do superimpose each other. The difference consists in the fact that no change will be observed in the area where both images overlap each other (neither brightness of the field of view will be affected at this point) if only the investigated object remains isotropic and homogeneous regarding its optical path. Fig. 6 explains this feature. Colouring of the field-of-view background (areas *a*, *e*) and that of space c where images B'_o and B'_e interfere with each other remains identi-

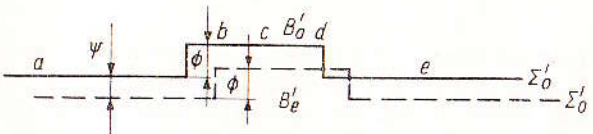

Fig. 6

cal as the optical path difference Ψ within those areas is identical as well.

Colours do change only in the areas *b* and *d* where images are doubled. The conditions within these areas remain the same as those in case of a totally doubled image, and this will only be here that the optical path difference Φ due to the object under examination will be measured.

Method discussed in the foregoing and hereinafter referred to as a *method of uniform colour with high image shearing* has been found of particular interest for observations of minor separate objects (like cells, bacteria, small microorganisms), thin layers and films being homogeneous in their character, fine crystals, etc.

A particular and the most interesting application of the uniform colour method in the polarizing interference microscopy appears to be the so-called *differential method* utilizing the fact that the transverse shearing of interfering waves becomes very small of an order of the microscope resolving power.

This method can be explained in the best way by referring it to a relatively large object showing a small gradient of the optical path difference, like e.g. a drop of liquid resting on the object slide (Fig 7a).

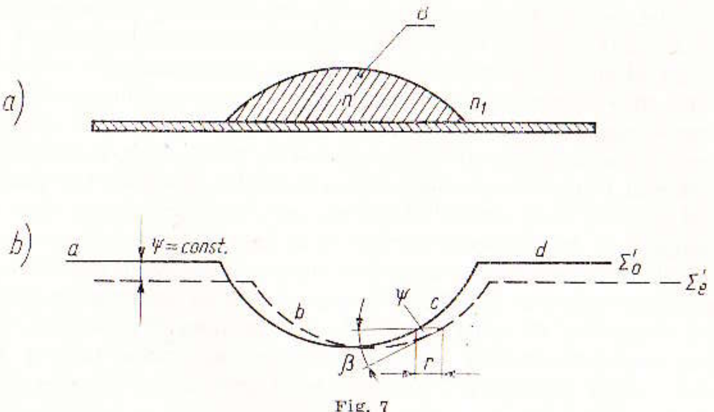

Fig. 7

Should refractive index of this object, n, exceed that of the surrounding medium, n_1, the front surfaces of interfering waves Σ'_o and Σ'_e will be formed as shown in Fig. 7b. As will be seen, optical path difference between waves Σ'_o and Σ'_e will remain constant only outside the image of observed object in the areas a and d where a uniform colour is produced. Within the image space, the optical path difference is not constant but will vary in a continuous manner thus involving also a continuous change in the colouring of image. With a symmetrical change in the optical path difference also colours in the observed object will be subject to a symmetrical variation versus those of the background whereby image centre has the same colour as the viewing field background.

A feature of the method under consideration, which differs it from other methods discussed before, basically consists in the fact that optical path difference Ψ in the interference image does not express optical path difference Φ within the object in a direct manner, but as a gradient of this difference towards the image shearing point. To explain this phenomenon in detail it will be necessary to make use of some mathematical formulae. Let us imagine to this end a rectangular coordinate system X, Y within image area of the object B (Fig. 8a) where X-axis runs towards the image shearing point (i.e. at right angles to refracting edge of the birefringent prism W_1) while Y-axis passes through vertex H of the object B. Let us now choose any x_1 point within the image B area. Optical path differenced Φ through the object B, with displacement dx on the X-axis, will now be as follows:

$$d\Phi=(n_1-n)dy \tag{6}$$

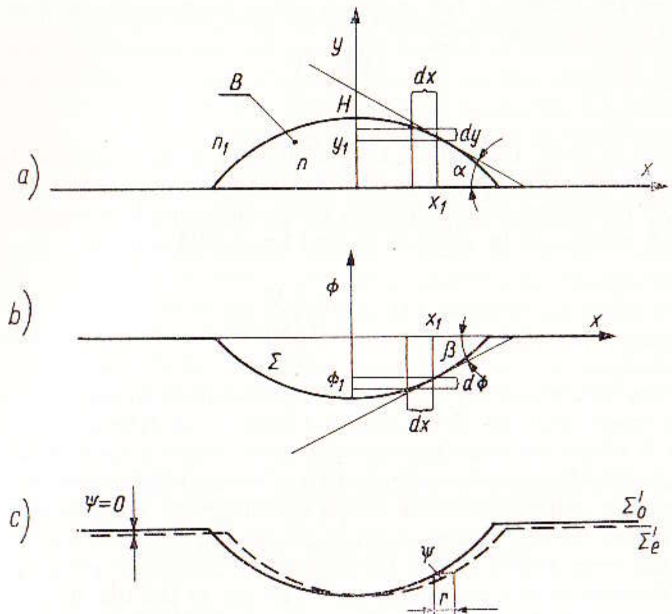

Fig. 8

As will be seen from Fig. 8a:

$$\frac{dy}{dx}=\tan\alpha \tag{7}$$

where α is an angle formed between the tangent passing through (x_1, y_1) and X-axis.

Hence:

$$d\Phi=(n_1-n)\tan\alpha\cdot dx \tag{8}$$

In other words:

$$\frac{d\Phi}{dx}=(n_1-n)\tan\alpha \tag{9}$$

On the other hand:

$$\frac{d\Phi}{dx}=\tan\beta \tag{10}$$

where β is an angle formed between tangent versus wave face at point x_1 (after light has passed through object B) and the X-axis (Fig. 8b).

The optical path difference Ψ between the split waves Σ'_o and Σ'_e at the point under examination (see Fig. 8c)

$$\Psi=r\cdot\tan\beta \tag{11}$$

where r forms transverse displacement of the interfering waves Σ'_o and Σ'_e (transverse separation).

From the formulae (10) and (11) taken together the following relation can be derived:

$$\frac{d\Phi}{dx} = \frac{\Psi}{r} \tag{12}$$

As to be seen, the optical path difference Ψ in a differential interference image represents a product of both the optical path difference gradient $d\Phi/dx$ and image shearing r. By comparing formulae (9) and (12) a new formula can be deduced to find tangential angle α:

$$\tan \alpha = \frac{\Psi}{r\,(n_1 - n)} \tag{13}$$

Hence, by measuring optical path difference Ψ at any desired point of the image one can find tangential angle α at relevant point of the object if only difference between refractive indices $n_1 - n$ is known. A few successive measurements of the optical path difference Ψ at some points will provide as a result object profile along any cross-section wanted. If the object profile is known, or if angle α at the given point has been found, it will be possible to determine either the difference between refractive indices $n_1 - n$, or one of the two indices, if only the other one is known. A measurement carried out for two different media of known refractive indices n_1, n_2 will provide in effect angle α and refractive index n. In such a case there are two equations of the formula (13) type from which both the refractive index n and $\tan \alpha$ can be found.

In addition, when one surface of the object under examination remains flat and the other is spherical it will be possible to find curvature R of such a surface after a previous measurement of both angle α and the distance x between apex of the spherical surface and the point at which the optical path difference Ψ has been measured:

$$R = x\,\frac{\sqrt{1 + \tan^2 \alpha}}{\tan \alpha} \tag{14}$$

Where the object being examined represents a ball having known radius R, the formulae (13) and (14) can be used to find one of the two refractive indices n and n_1 if only the other refractive index remains known.

The optical path difference Ψ within the object under investigation can be found from a change noted in colour, or else may accurately be measured by using the formerly described method of darkening the given point of image and establishing transversal shift of the birefringent prism, performed during this step. For more details of this measuring technique see Chapter 5.

As seen from the foregoing, the differential method offers much more in the sphere of measurement than said method of uniform colour with high image shearing effect. This is, however, not the only advantage to be gained from that method. In addition to numerous applications in the field of quantitative research the differential method offers also some more extremely interesting potentials for the qualitative research. The differential interference image stands out, as a matter of fact, for its high degree of plasticity (in the stereoscopic meaning of the word) and fidelity of representation. Moreover, such an image remains free from the harmful halo effects and other undesirable imperfections typical for the phase-contrast technique. The differential image provides also much more information about the shape, structure and appearance of various objects (both phase and amplitude ones) as compared with the phase contrast microscopy.

2. PRINCIPLE OF OPERATION FOR A POLARIZING INTERFERENCE MICROSCOPE INCLUDING TWO BIREFRINGENT PRISMS

(Application of Shearing Objectives)

The range of applications for a polarizing interference microscope with one birefringent prism in the event of uniform colour method including high image shearing effect (prism No. 3) is confined to the examination of objects having relatively small transverse dimensions, such as biological cells, thin fibres, or such extensile specimens whose edges are steep and which are uniform in their optical path difference.

A far greater image shearing (doubling) in a uniform interference field is possible due to the substitution of ordinary standard objectives, forming part of normal microscope outfit, by the high shearing objectives (or polarizing interference objectives). The introduction of high shearing objectives changes the microscope into a polarizing interference unit whose schematic diagram is presented in Fig. 9.

Prism W_1 (in the interference head) and prism W_2 (in the objective) form, together with the crossed or parallel polaroids P and A and the slit S located in the condenser K focus and disposed so as to be parallel to refracting edge of the prism W_1, a kind of double polarizing interferometer. Prism W_2 having a relatively large angle of refraction is located right behind the last lens of objective Ob and can be swung about the objective centre line, with plane H_2 in which interference fringes of that prism are being formed coinciding with focus F' of the Ob objective. Another birefringent prism W_1, having a much smaller angle of refraction φ_1, is installed in the interference head.

Angular motion of the W_2 prism serves for the adjustment of image shearing values. If this prism remains set so that its angle of refraction φ_2 has the same orientation as angle of refraction φ_1 in prism W_1,

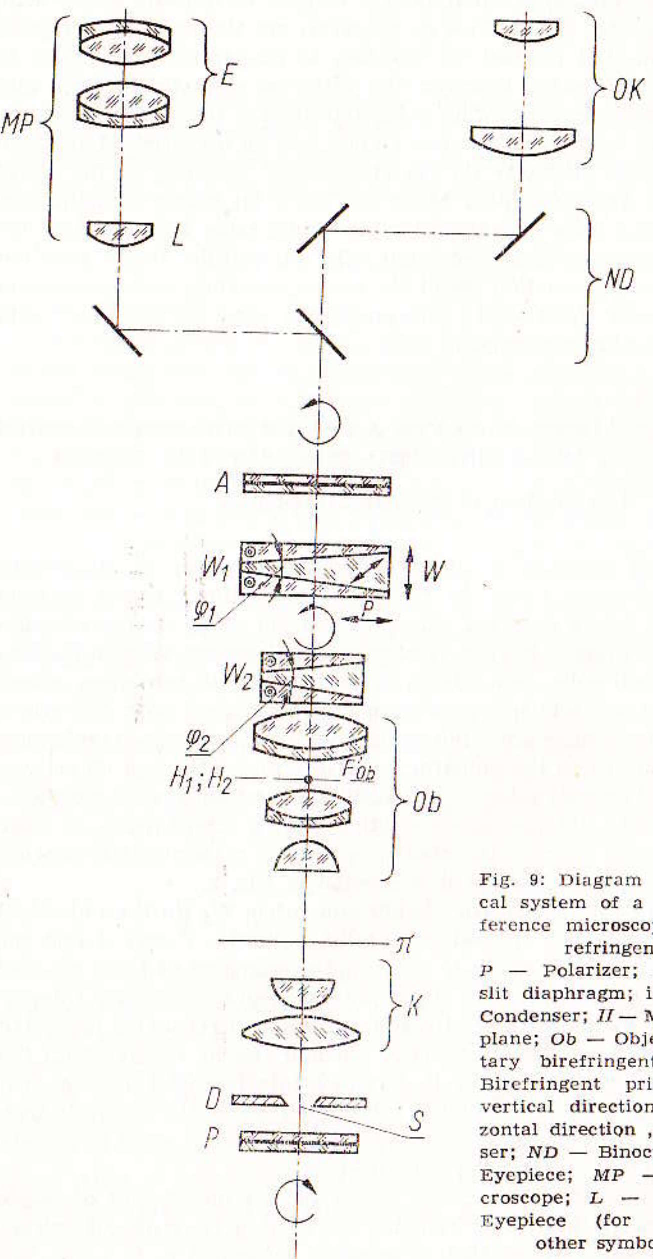

Fig. 9: Diagram illustrating optical system of a polarizing interference microscope with two birefringent prisms
P — Polarizer; D — Condenser slit diaphragm; incl. slit S; K — Condenser; Π — Microscope object plane; Ob — Objective; W_2 — Rotary birefringent prism; W_1 — Birefringent prism slidable in vertical direction „w" and horizontal direction „p"; A — Analyser; ND — Binocular body; Ok — Eyepiece; MP — Auxiliary microscope; L — Objective; E — Eyepiece (for explanation of other symbols see text)

then a maximum image shearing r — being the sum of shearings r_1 and r_2 produced by each prism separately — will be the final effect. If, on the other hand, prism W_2 has such an orientation that its angle of refraction φ_2 remains reversed versus angle of refraction φ_1 in prism W_1, then the resultant image shearing r will be equal to the difference existing between shearings r_2 and r_1. In an intermediate position when refracting edge of prism W_2 forms an angle of 45 degrees versus this of the W_1 prism, the resulting image shearing r will be equal to shearing r_1 to be produced only by prism W_1. In this case, the system can be referred to this shown in Fig. 1.

It follows then that by rotation of prism W_2 about the Ob objective centre line three different image shearing values, i.e. r_2+r_1; r; and r_2-r_1, can be found. In these circumstances, it will be possible to select the required image shearing effect quickly and so as to suit actual width of the object under investigation.
Without the need for any objective or birefringent prism replacements as this is usual for the polarizing interference microscopy with only one birefringent prism in operation. The maximum shearing values produced in such a way with objectives of different magnifying powers can be several times greater thus enabling the measurement of optical path difference to be carried out with regard to extensile items, broad biological cells, and even biological sections.

Where use is being made of fringe prism No. 2 (W_1), with prism W_2 having an opposite orientation versus the former (φ_1 reversed versus φ_2), an interference fringed field with a differential image shearing is the outcome of the procedure. Such a possibility does not exist in the optical system employing one birefringent prism only. By shifting fringe prism No. 2 in the vertical direction small differences can be produced in the image shearings, caused by both prisms, and so optimum conditions of observation and measurement can be derived according to the actual width of the item under examination and optical path difference gradients occurring within such an item.

The method of differential interference and fringed field appears to be particularly suitable for the measurement of birefringence in the event of birefringent fibres and films, especially where such fibres and films do exhibit some minor local heterogeneities.

By rotating prism W_2 through 180 deg. so as to make its angle of refraction φ_2 coincide with that of fringe prism No. 2, a high image shearing effect, such as to enable measurement of the optical path difference for both isotropic as well as anisotropic objects along with their thickness, refractive indices and other physical quantities, can be produced.

The availability of a complete set of objectives and birefringent prisms W_2 renders that microscopic examination can be effected using one of the following interference methods:

tions, viz. in the parallel direction versus microscope centre line and at right angles thereto.

Parallel displacement requires that a knurled ring *14*, provided on the head perimeter, is put into action. Vertical movement is achieved through rotation of the micrometer screw head *15* extending to the side. Thimble of the micrometer screw carries a scale with 0,01 mm graduations to enable measurement of the transverse movement of prism.

Parallel adjustment versus optical axis of the microscope (vertical movement) produces a uniform interference field (prisms Nos. 1 and 3) followed by changes in the width of fringes observed in the field of view (prism No. 2) whereas perpendicular adjustment serves for control of phases between the interfering ordinary and extraordinary light waves and for the measurement of optical path difference in the object under observation.

Measuring range for the differential prism (No. 1) is unilateral, being bilateral for the other ones. This means that the differential prism (No. 1) is allowed to move from its zero position (dark background of the field of view) to a greater extent in one direction only whereas the uniform colour prism (No. 3) and the fringe-producing one (No. 2) are movable in both directions (i.e. left and right from zero position).

Analyser enclosed by housing *16* rests in the upper part of the interference head. Housing of the analyser can be rotated through 360 deg. and the analyser itself through 90 deg. On the half of the mount circumference a scale is marked in degrees every 15° from 0 to 90 deg. Opposite this scale a red dot is provided to indicate the out-of-light-beam position of the analyser.

To read the actual position of analyser make use of a notch provided in the upper plate serving as a guard for the analyser.

Condenser with Slit Diaphragm (Fig. 12) represents in fact an Abbe type condenser finding normally use in the microscopy.

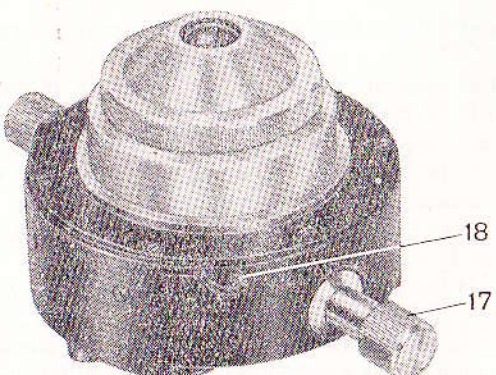

Fig. 12: Condenser with slit
17 — Slit with adjustment; *18* — Slit length limiters

A slit mechanism is permanently connected with the enclosure of the condenser. Slit diaphragm is formed of two jaws mounted in relevant carrying slides. These jaws can be moved independently from each other by means of a pair of controls *17* so as to take any lateral position and to decentre the slit, if required, for getting an oblique beam of light.

The maximum possible separation of jaws is approximately 15 mm. This distance makes it possible to conduct the observation in a normal bright field with condenser aperture increased versus this existing at the slit illumination conditions.

Slit length can be adjusted by means of two symmetrically disposed diaphragms *18*. In the bottom part of the jaw assembly provision has been made for guides to mount the polarizer fixed in its position through a ball click.

Polarizer (Fig. 13) represents a kind of polarizing filter cemented into a rotatable ring with the angular scale. This scale has incisions made every *5* degrees in the two opposite directions from 0° to 180° and has a two-sided vernier *20* and *21* to permit angular position of the polarizer for being read with 1° accuracy.

In addition, two symbols „x" and „||" have been marked on the scale between numbers 40—50 and 130—140. These denote crossed „x" or parallel „||" position of the polarizer in relation to analyser set to „45". The direction of light oscillation coincides with that of the 0—180° ring diameter.

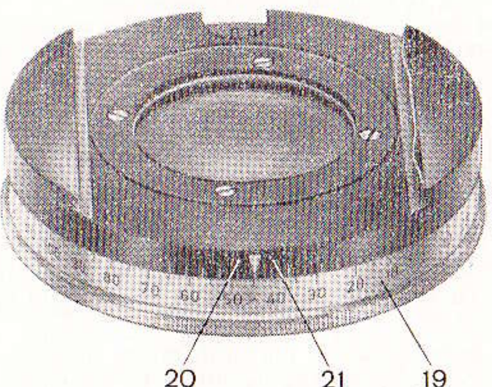

Fig. 13: Polarizer
19 — Angular scale; *20* — Left-hand vernier (nonius); *21* — Right-hand vernier (nonius)

Condenser with compensators (Fig. 14) differs from the former one in that instead of a slit diaphragm it incorporates a system of four quartz compensators suitably disposed in a revolving disk *22*. Each compensator has been adapted for use with one of the objectives from the set, i.e. ×10, ×20, ×40, or ×100.

On the revolving disk housing there are marked numerals 10, 20, 40 and 100, with a fixed index being provided on the stationary part of the housing.

A perceptibly locking spring-loaded click is an ease in bringing the required numeral in front of the index. Number 20, when in one line with the index, denotes e.g. that compensator for the ×20 objective has been engaged.

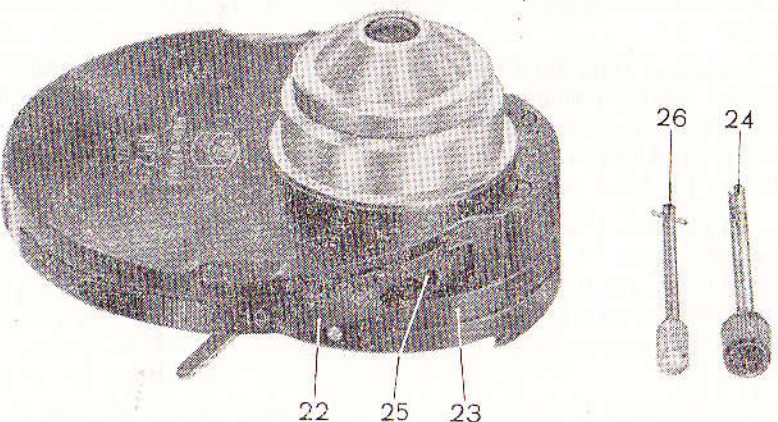

Fig. 14: Condenser with compensators
22 — Revolving disc; *23* — Iris diaphragm; *24* — Key foı adjustment of compensators; *25* — Key hole; *26* — Key for tightening condenser with the condenser holder

Each compensator is rotatable within ±10 degrees which operation can be performed by the aid of relevant adjustment. To achieve the required setting use must be made of key *24* which through opening *25* fits the adjustment screw to positions locked by the click.

In addition to compensators there is still another free opening provided in the revolving disk (marked „0" on the disk circumference) and designed to carry on observations in normal bright field or polarized light.

A small key *26* forming part of the outfit serves for tightening clamping screw of the condenser during its fitting in the microscope substage seat.

The polarizer (Fig. 13) connects with the condenser incorporating compensators in an identical manner as with the slit diaphragm condenser.

Auxiliary Microscope (Fig. 15) has been designed to watch exit lens of the objective and to adjust the microscope with the polarizing interference attachment. It finds use in the generally known phase-contrast microscopy.

Eyepiece *30* is made to move in the objective tube *28*. Incisions in the bottom part of eyepiece tube serve the purpose of eliminating clearances between tubes by such a deflection of the tab as to render any fortuitous change in the position of tube impossible.

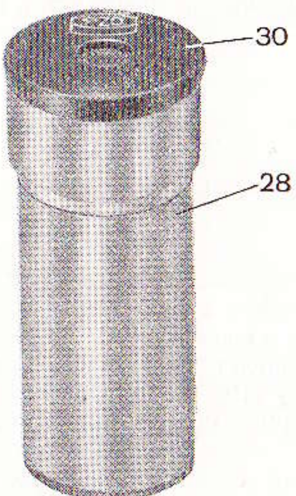

Fig. 15: Auxiliary microscope
28 — Objective; *30* — Eyepiece

Interference Filters 5 (Fig. 10) are intended for the monochromatic light observations. There are two filters which are supplied as standard accessories, a green filter FI 546 and a yellow one FI 590.

Maximum transmittance for the first of them coincides with the mercury green line (λ=546 nm), and with the sodium yellow line (λ=589 nm) for the second. The half-wave bandwidth of the transmittance for these filters ranges to about 1 nm, with the transmittance itself exceeding 35%.

Polarizing Interference Objectives — As regards optical system of the polarizing interference objective, referred also to as a high image splitting objective, differs from that of an achromatic one in that it has a birefringent prism installed therein. The prism is rotatable together with the objective optical system through 360° versus a knurled mount which fits objective nose piece being screwed in into it.

A complete set includes objectives of the following magnifying powers: ×10; ×20; ×40; and ×100. These are used exclusively with a slit

diaphragm condenser. Birefringent prisms of said objectives provide the following r_2 values of image splitting (in relation to the object plane):

40 μm for ×10 objectives;
20 μm for ×20 objectives;
12 μm for ×40 objectives;
5 μm for ×100 objectives.

The polarizing interference objectives are marked both with a red strip and with letters „PI".

Measuring × 12 Eyepiece (Fig. 10) is very useful in finding the inter-fringe spacing h for a fringe-producing prism (No. 2).

Chapter 3

PREPARING A MICROSCOPE FOR NORMAL USE

Precision in the adjustment of a polarizing interference microscope is a prerequisite to achieve a good-quality image and to arrive at the best possible results of a measurement.

Accordingly, preparation of a microscope for observations and measurements to be made by different techniques should proceed with a due care and without simplifications in full conformity with directions as detailed below.

The most correct and preferable setting of a microscope is that shown in Fig. 16.

The first step consists in a connection formed between the interference head and microscope limb by means of a clamp *8*. Analyser *16* (Fig. 11) should be mounted so as to face the observer. A stud protruding from the lower interference head plate must be aligned so as to engage a groove specially made in the clamp mount to receive it and to prevent thus any inadvertent movement of the interference head.

The next step includes location of the eyepiece *31* in the interference head seat and positioning it with the aid of a clamping screw *32*.

The slit diaphragm condenser *2* is to be mounted in the microscope substage condenser *33* (Fig. 17) so as to have the ball click, provided in the lower part of slit mechanism, directed towards the microscope limb. The condenser remains in its mount immobilized by means of a clamping screw *34* (Fig. 17).

The condenser and compensators *3* (Fig. 18) can be installed similarly to that with a slit diaphragm. The only difference consists in using a special small key *7* (Fig. 18) to tighten screw *34* (Fig. 17). A symmetrical positioning of the condenser in relation to substage will be of primary importance.

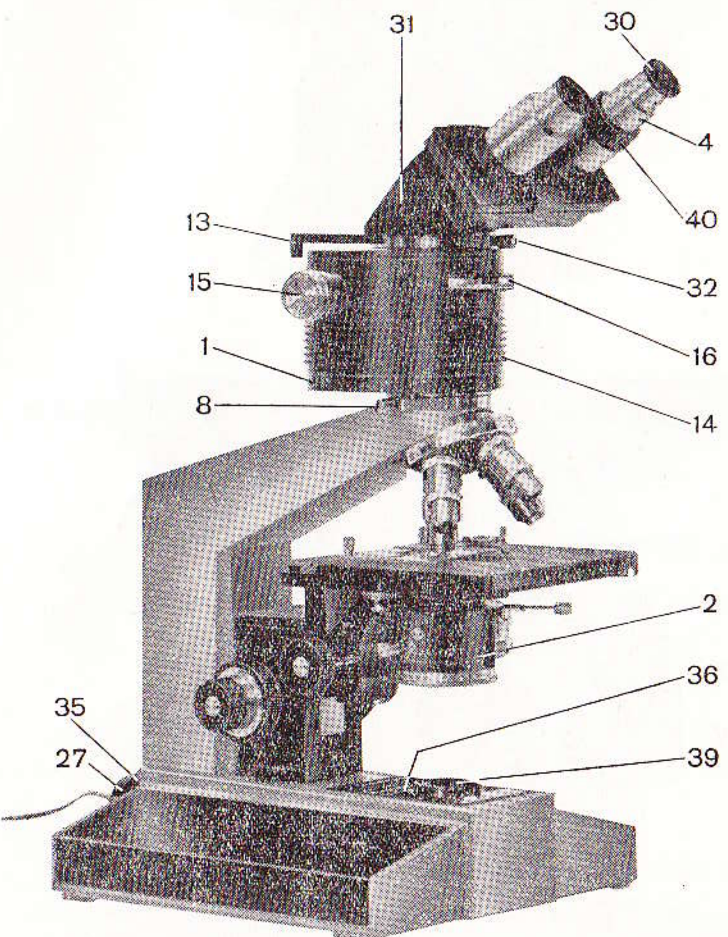

Fig. 16: BIOLAR PI Polarizing Interference Microscope (Condenser with slit)

1 — Interference head; *2* — Condenser with slit; *4* — Auxiliary microscope; *8* — Clamping screw; *13* — Prism operating lever; *14* — Knurled ring for parallel adjustment of prisms in relation to optical axis; *15* — Micrometer screw for perpendicular adjustment of prisms in relation to optical axis; *16* — Analyser in its housing; *27* — Lamp fitting; *30* — Auxiliary microscope eyepiece; *31* — Binocular body; *32* — Binocular body clamping screw; *35* — Illuminator; *36* — Field diaphragm adjustment; *39* — Filter seat; *40* — Binocular tube dioptric ring

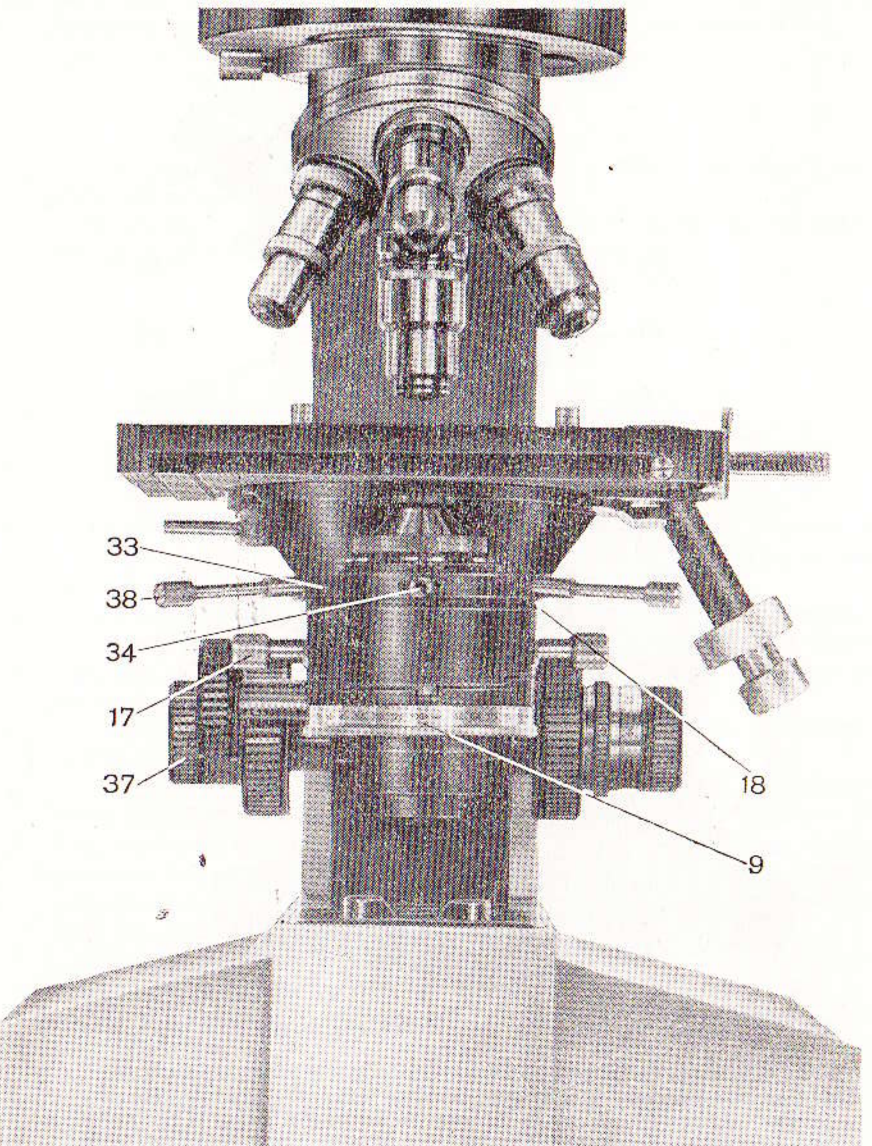

Fig. 17: Lower part of the microscope with slit diaphragm condenser mounted thereon

9 — Polarizer; 17 — Slit with adjustment; 18 — Slit length limiters; 33 — Condenser lift mount; 34 — Condenser clamping screw; 37 — Condenser adjustment; 38 — Condenser control screws

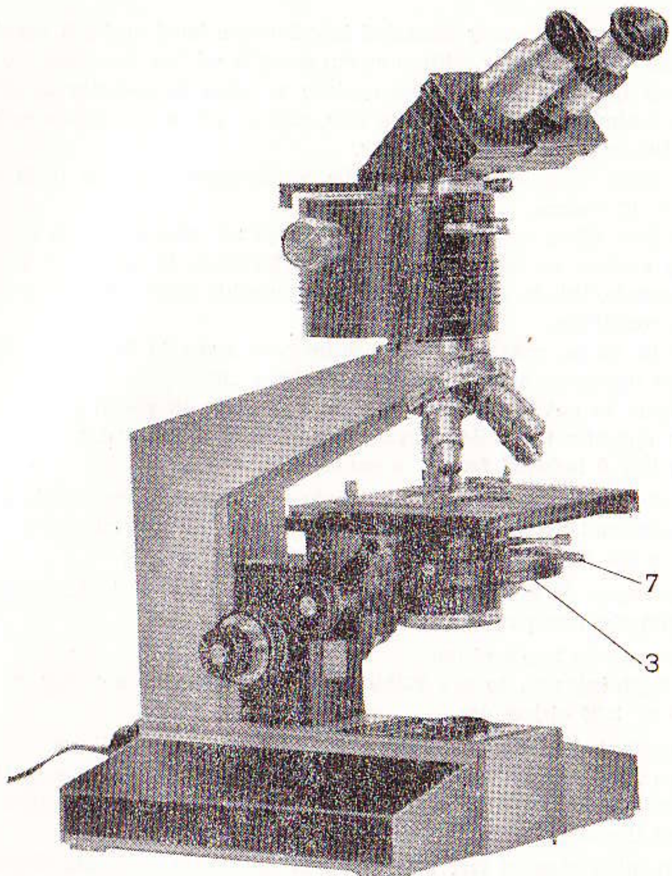

Fig. 18: BIOLAR PI Polarizing Interference Microscope (Condenser with compensators)

3 — Condenser with compensators; 7 — Key for tightening condenser with compensators in its holder

Condenser with the compensators finds use only in conjunction with a differential prism (No. 1) and serves exclusively for the purpose of qualitative investigations where high aperture and much light are required.

For quantitative examinations and measurements only the slit diaphragm condensers are to be used since the so-called „aperture error" may otherwise be the case due to a high aperture characteristic for the condensers with compensators.

1. ILLUMINATION SETTING PROCEDURE

1.1. With the properly installed interference head and slit diaphragm condenser (or condenser with compensators) it will be necessary to screw objectives into the objective no sepiece, to slide illuminator *35* (Fig. 16) into its socket in the microscope foot, and to place respective eyepieces in the binocular attachment tubes.

The lamp is to be connected via a microscope power pack to the 220 V A. C. mains.

The first thing to be done in thus prepared microscope is to set the lighting system in conformity with the Köhler's illumination principle requirements, this to ensure the most favourable observation and measurement conditions.

At this stage, particular accessories will have to be set as follows:

a) prism changing-over lever *13* in position „0";
b) analyser *16* out of operation, i.e. in a position in which a dot is to be seen opposite the index having the form of a sighting notch;
c) polarizer *9* isolated from the condenser;
d) slit diaphragm jaws drawn to their extreme positions aside (this to be achieved by operating screws *17* clockwise till resistance) or disc of the condenser with compensators in position „0";
e) diaphragms *18* in their extreme OPEN positions, with the front lever moved right and rear one left;
f) condenser in top position.

1.2. Lighting acc. to the Köhler's principle can be set best by using ×10 or ×20 objectives.

The adopted sequence of steps should then be, as follows;

a) Place specimen on the object stage; switch on the light and by first operating macro and then also the micro (coarse and fine) adjustment bring the image into focus;
b) By turning ring *36* left till resistance shut next the field diaphragm, and by manipulating control *37* (Fig. 17) move the condenser so as to focus diaphragm edges in the field-of-view plane; if the image remains out of centre in the observed field of view, its central position will be restored by making use of the adjustment screws *38*.
c) Open field diaphragm so as to completely uncover the field of view; any further continuation of the opening process is not advisable since reflections reducing the image contrast are then likely to be produced.
d) In place of one of the eyepieces insert the auxiliary microscope (Fig. 16) No. 4.
e) By using the auxiliary microscope move eyepiece *30* until a sharp image of the objective exit lens is produced (a bright circle of the sharply represented contours to be focused in the field-of-view plane).

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f) Move illuminator *35* with the lamp in its foot socket to a position in which image of the lamp filament will be in focus when viewing through the auxiliary microscope.
g) By applying a swinging motion to the lamp fitting bring the seen filament image into central position of the auxiliary microscope field of view.
h) By turning screws *17* of the slit diaphragm condenser there and back (Fig. 17) readjust jaws so as to dispose same symmetrically versus lens centre spaced some 3 to 5 mm far in the apparent field of view.
i) Replace polarizer by sliding it onto condenser guides.
j) In the event of condenser with compensators omit steps e, f, g, and h, and while observing from underneath the plane of condenser iris diaphragm move lamp in the foot seat so as to obtain a centrally disposed sharp image of the filament seen in the iris diaphragm plane. With the steps as above duly completed proceed with the adjustment proper. This will differ according to the adopted observation and measurement technique.

2. DIFFERENTIAL METHOD — SLIT DIAPHRAGM CONDENSER

2.1. Set lever *13* (Fig. 11) to position "1".

2.2. Shift specimen (by moving the object stage) so as to locate the objective outside the investigated specimen area where no significant optical heterogeneities are encountered.

2.3. Set analyser *16* to scale mark "45" with the polarizer simultaneously moved to "45" or "135" ("x" position).

By viewing through the auxiliary microscope *4* (Fig. 16) and widening the slit a little with the aid of controls *17* (Fig. 17) one can notice in the objective exit lens broad interference fringes together with a dark zero order and coloured fringes of the first, second and further interference orders.

The system of interference fringes and a dark zero fringe comes into existence when the polaroids remain crossed (analyser *16* set to "45" and polarizer *9* to "x"), and this with a bright zero fringe when the polaroids are parallel (analyser set to „45" and polarizer to „‖").

Only for these positions of the polarizer *9* maximum intensity of fringes can be had in the objective lens together with a best possible contrast obtained for the image of the object under examination.

The choice of this or another system of interference fringes will depend upon the kind of specimen being observed, and upon the adopted investigation programme. Normally, the system of fringes with the dark zero fringe is to be regarded as prefereable.

2.4. By operating controls *17* (Fig. 17) narrow the slit so that its width seen in the field of view be approximately 2 mm, and readjust it so as to produce its image disposed centrally in the objective exit lens (when observed through the auxiliary microscope) and in parallel with the direction of interference fringes.

Fig. 19: Objective exit lens
a — Slit image; *b* — Birefringent prism refracting edge; *c* — Objective exit lens; *d* — Direction of light oscillations in analyser; *e* — Direction of light oscillations in polarizer

Should the slit image lack the parallel alignment versus the direction of zero fringe, then the slit diaphragm condenser would have to be a little twisted in the substage condenser mount *33*. To do this, slightly loosen condenser clamping screw *34* while holding the very condenser with hand to prevent its fall down. Proceed with tightening it more firmly no sooner than the slit direction has been precisely fixed. Then, establish the required slit width so that only the purple colour be cut out of the first colour fringe (when counting from the dark zero fringe). This fringe is to be brought down to be in one line with the slit image, which operation is to be completed with the birefringent prism shifted by means of control *15* (Fig. 16).

Now, by making use of diaphragms *18* (Fig. 17) reduce the slit in its length to such an extent as to keep the dimension of the fringe being cut out within the confines of that of the objective exit lens diameter.

A mutual orientation of the slit image and interference fringes located in the objective exit lens versus the direction of light oscillations in the polarizer has been show in Fig. 19.

2.5. If the slit exactly coincides with the purple portion of the interference fringe, the field of view as observed through the eyepiece should remain also completely purple (Fig. 46b) by having the same colour as that "cut out" of the interference fringe by the slit.

Should the field of view lack such a complete purple colouration, with some other adjacent colours (Fig. 46a) being yet present in addition to the purple colour, the conclusion will have to be that the birefringent prism is not at an appropriate distance versus the objective focus. In such a case, the knurled ring *14* (Fig. 16) must be rotated this or another way until a uniformly purple field of view is produced. During the operation of ring *14*, the birefringent prism may slightly move aside thus causing fading of the purple colour.

Said colour can be restored by operation of control *15*. Special attention must also be given at this stage (particularly when an x10 objective is used) that the image of lamp filament does not coincide with the object plane so as to interfere with the field-of-view uniformity. Should this be the case, the lamp will have to be slightly moved in this or another direction until a possibly uniform field of view is achieved. In some instances, the lack of uniformity caused by the filament can be removed by means of a ground glass to be placed in the microscope foot seat *39* (Fig. 16).

To raise the birefringent prism rotate knurled ring 14 in the clockwise direction, and to lower it a reverse sense of operation is to be applied.

2.6. By controlling substage position bring the specimen in line with the objective field of view, select the required fragment and use fine adjustment to produce a sharp image.

2.7. Remove auxiliary microscope 4 and install in its place a second eyepiece. Readjust eyepiece spacing according to viewer's needs and then rectify sharpness of the image by viewing first with one eye only through the fixed eyepiece and subsequently by operating the diopter correction collar 40 (Fig. 16) of the tube to match movable eyepiece for distinct vision of the second eye. This is a routine usually required when a binocular eyepiece is used.

2.8. With the above steps duly completed, the microscope with a polarizing interference facility can be regarded as ready for use including observations, measurements or microphotography. When changing to an other objective, condenser diaphragms will have to be checked at jaws drawn apart and illumination setting verified with the analyser out of operation by following items 1, 2 (a, b, c) and by completing operations described in Its. 2.4.—2.7.

With so prepared microscope and the uniform field of view coloured purple (first order colour), a similarly uniform field of view will be automatically obtained for any other interference colour.

At one extreme position of the birefringent prism a dark interference field is produced whereas the other extreme position results in an interference contrast of the second and third interference order colours. By setting the birefringent prism to dark field of view, and then by moving it in the opposite direction, an interference contrast in the grey and grey-green field is the outcome (in such a field, the image becomes to some extent similar to a phase-contrast image being, however, much more plastic and without any harmful halo effects characteristic for the phase contrast).

Next, a bright field comes to be seen (the image of phase objects looses on its contrast or becomes hardly visible at all); further on, a yellow colour (providing a rather good contrast) and then orange, purple (sensitive), blue and green appear on the scene.

In between these colours a wide range of transitional colours of various shades is produced. A further shifting of the birefringent prism results in the formation of identical colours of the second and third interference order but with a slightly different hue generally providing a worsened contrast of the microscopic image. Within these colours it is possible to select the best conditions of observation for the specimen to be analysed (good contrast, adequate plasticity and distinct contours of details).

By rotating polarizer *9* (Fig. 17) in either direction through 90°, i. e. so as to bring it into position marked "‖", observations can be automatically performed with the polarization planes of the polarizer and analyser *16* (Fig. 16) set in parallel versus each other. A dark field is to be expected in this specific case. Colours within the specific interference orders resemble those characteristic for the crossed polarizer and analyser, but their hue is a little with quite good observation prospects in many an instance.

If one wishes to get quickly a comparison between the nature of image produced by means of a polarizing interference microscope and that by means of an ordinary standard (bright field) microscope, it will be sufficient to disengage the analyser or to twist the polarizer through 45° from its "x" or "‖" position.

3. UNIFORM FIELD METHOD INCLUDING HIGH IMAGE SHEARING EFFECT

With the microscope including a slit diaphragm set in conformity with It. 2 shift lever *13* (Fig. 16) to position "3".

Microscope adjustment process proceeds in this case similarly as that in the event of a differential method. With the microscope readjusted to

the uniform field observations, a dark field will be produced (at crossed polarizer and analyser) with the birefringent prism set more or less to its central position. When moving the prism in either direction versus this position, several uniform colours will arise of the first, second, third and subsequent interference orders.

Measurements and observations will have to be carried out as near as possible to the field of view considering the possibility that a certain loss of colour uniformity is likely to occur on field borders.

A microscope with binocular eyepieces is to be employed in such a case.

4. FRINGE METHOD

4.1. Readjust lighting in conformity with the Köhler's illumination principle (It. 1) and then shift lever *13* (Fig. 16) into position "2".

4.2. By adequate movements of the substage position the specimen so as to have the objective outside the limits of the examined specimen area i.e. in the location where no major optical heterogeneities can be experienced on the image.

4.3. Set analyser, as formerly, to "45" and polarizer to "45" or "135" marked by "x".

4.4. Make the slit diaphragm as narrow as possible, with a due attention given at the same time to its central position versus optical axis of the microscope.

A split image of the slit, when viewed through the microscope, ought to be disposed centrally versus the objective exit lens.

4.5 Rectilinear interference colour fringes (Fig. 45), including a dark zero fringe, ought to be seen when looking through the eyepiece. If this is not so, move the birefringent prism in its transverse direction (by making use of control *15*) until fringes are to be seen in the microscopic field of view.

Subsequently, with the slightly tightened clamping screw *34* (Fig. 17), rotate condenser in its seat this or other way by operating one of the controls *17* until interference fringes get their maximum clarity and an appropriate brightness, at the same time, in the microscopic field of view is produced.

4.6. With all the above steps brought to an end, the microscope can be regarded as ready for commencing the field fringe observations. When changing from one objective to another it will be only necessary to rectify accordingly width of the slit and opening angle of the field diaphragm and/or central positioning of the condenser. It would be also worth noting that while moving the birefringent prism along microscope axis (ring *14*), the interference can be to some extent broadened or narrowed. With the birefringent prism in its upper position the fringes

become narrower, and broader in its lower position. Measurements are, nevertheless, recommended to be taken at the lowest position of the birefringent prism.

Approximate values of the constant p' in Table **II** exactly apply to this position. Constant p' is to be found each time a higher precision of measurement will be required.

5. DIFFERENTIAL METHOD — CONDENSER WITH COMPENSATORS

5.1. Mount the condenser with compensators in the lifter seat. Slide polarizer (Fig. 13) upon the condenser guides. Condenser itself is to be arranged symmetrically versus adjustment screws *38* (Fig. 17). Clamping screw *34* is to be tightened with a small key *7* (Fig. 18). Analyser *16* (Fig. 16) is to remain as set before, i.e. adjusted to "45", and polarizer *9* (Fig. 17) to „45" or „135", marked „x".

5.2. Produce lighting in accordance with the Köhler's illumination principle (see It. 1) so that the revolving disc is in its "0" position (fully open port).

Observation is to be carried out using a ×10 objective.

5.3. By operating revolving disc *22* (Fig. 14) cause the compensator "10" to enter the beam of rays.

5.4. While observing exit lens of the objective through an auxiliary microscope use control *15* to produce purple (sensitive) colour of the first interference order. Image of the lamp filament in the lens ought to be filled with this colour. Should it happen otherwise so that still some other adjacent colours be visible in the field, condenser in its substage holder *33* (Fig. 17) will have to be rotated this or other way.

Rotation is to be effected no sooner than the clamping screw *34* has been loosened a little with the lower part of the condenser held firmly with the other hand.

Compensators are factory adjusted and therefore no additional adjustment (condenser manipulation) after an objective has been replaced is required, with the condenser brought out of adjustment, i.e. when no such a condition has been satisfied, the position of compensators must be rectified with the use of key *24* (Fig. 14) to be inserted in seat *25*.

Once adjusted, the compensators do not call as a rule for a readjustment when reusing he condenser provided the latter has always one and the same position in its holder.

5.5. If the colour of filament image in the lens remains uniform, then the field of view as seen through the microscope eyepiece will be also uniform having the same colouration as the exit lens of objective.

Should it be different, the birefringent prism will have to be brought farer from the objective as prescribed in It. 2.5.

5.6. With all the steps above duly completed, the microscope can be regarded as fit for operation.

Further operations should proceed to Its. 2.6. and 2.7. When changing from one objective to another one, relevant compensator will have to be engaged and steps as referred to in Its. 5.2. and 5.5. repeated. An iris diaphragm *23* (Fig. 14) located under the condenser serves the purpose of narrowing condenser aperture, this applying in particular to the plain „bright field" microscopy with revolving disc *22* set to „0".

6. APPLICATION OF POLARIZING INTERFERENCE OBJECTIVES

6.1. Differential and Uniform Colour Methods

The microscope is to be now set in accordance with the principles described above and with either No. 1 or No. 3 birefringent prism put into action according to actual needs. Then, a birefringent prism objective is to be provided in place of a standard one and its setting must be such (by operation of its mount) as to see the interference fringes, viewed through an auxiliary microscope, to run parallelly with those of the prism installed in the interference head. In such a case, the two patterns of interference fringes produced by birefringent prisms in action coincide with each other thus producing a new pattern of more dense and narrower fringes.

Subsequently, condenser slit should be narrowed so as to „cut out" of this resultant pattern the first order purple colour. In these circumstances, the field of view should remain uniform in its colour. If this fails to be so, slightly raise or lower the prism in the interference head by operating knurled ring *14* (Fig. 11).

The objective ought to be set so as to have a sharply contoured image of the specimen. With this being so, rotate objective mount with the birefringent prism installed therein through 180° or 45° to obtain other image splitting values.

Should, however, the field of view lack its uniformity after said operation, its initial condition will have to be restored by means of the knurled ring *14* operated in either of the two directions.

6.2. Fringe Method

The microscope is to be set in conformity with the principles specified above and a birefringent prism objective provided in place of a standard one. Now, operate objective mount so as to produce in the field of view the maximum distinction and best contrast interference fringes, and adjust the width of slit accordingly.

Chapter 4

CALIBRATION OF BIREFRINGENT PRISMS

1. DETERMINATION OF THE INTER-FRINGE SPACING

The inter-fringe spacing h represents a characteristic quantity of the birefringent prism W_1 provided in the interference head. Prism W_2 in the objective remains without effect on the h value.

1.1. Finding Inter-Fringe Spacing h for Differential Prism (No. 1) and Uniform Field Prism with High Image Splitting Effect (No. 3).

The microscope must be precisely focused and set to a uniform-coloured field of view.

Where monochromatic light with an installed interference filter is employed, the measurement of interfringe spacing h for birefringent prisms providing an uniform field of view will consist in the reading of micrometer screw thimble indications to find a shift in the prism being equivalent with the two successively following maximum darkenings of the field of view.

To achieve a higher degree of accuracy it is recommended to read the difference between the possibly two most extreme positions of the prism where still another dark field of view is produced and to divide the read position difference by the number of successive darkenings of the field of view less one (generally speaking, the difference of prism positions corresponding to the first and n-th darkenings of the field of view is to be divided by the number of darkenings amounting to $n-1$).

Let us assume that e.g. W_1 represents prism position in which the field of view is for the first time completely darkened (maximum darkening), and W_5 another prism position in which the field of view becomes for the fifth time maximally darkened (with the prism travelling all the time in one direction only), then the inter-fringe spacing will be $h = \frac{W_5 - W_1}{4}\ \mu m$.

Another procedure includes a method according to which position of the birefringent prism is being read for each maximum darkening of the field of view to find next a mean value of the diefferences between successive prism positions.

When white light is used, calibration of the uniform field prisms proceeds so that the birefringent prism is set to the dark field of view and then to the first order sensitive colour. The amount of birefringent prism shift occurring during this operation and read from the micrometer screw thimbles is right the inter-fringe spacing h that is looked for. It is also possible to utilize here very sensitive colours of the second and third interference order (on both sides of the dark zero fringe) while finding a mean value from differences between successive prism positions in which a purple field of view is produced.

1.2. Finding Inter-Fringe Spacing h for a Birefringent Prism (No. 2)

Somewhat different is the calibration of a birefringent prism providing the fringed field of view. In such a case, an ×12 measuring eyepiece is inserted in place of a standard eyepiece in the binocular attachment and the prism is lowered into its bottom position, the latter operation is effected with ring *14*. Now, by moving the birefringent prism in the transverse direction, centres of interference fringes translocating in the field of view are being set to one of the freely chosen lines of the eyepiece scale with the successive positions of the birefringent prism simultaneously read from the micrometer screw settings. A mean value taken, say, from 10 successive birefringent prism settings gives the interfringe spacing h. Interference fringes remote from the zero fringe should be avoided. It would be preferable to restrict oneself to only five fringes being taken from each side of the zero fringe. Whenever the white light is used, it will be the point where violet changes into blue that should be regarded as a centre of colour fringes.

Table II shows the inter-fringe spacing values h for various positions of the birefringent prisms in the white and monochromatic light observations with wavelengths equal to $\lambda=590$ nm and $\lambda=546$ nm respectively. These are theoretical values. For practical reasons, these values are recommended to be determined under the true conditions of investigation.

2. DETERMINATION OF THE CONSTANT p'

Constant p' is to be known if one has to measure the fringed field optical path difference (prism No. 2) by using the method of birefringent prism shift described herein. To find this value it is recommended

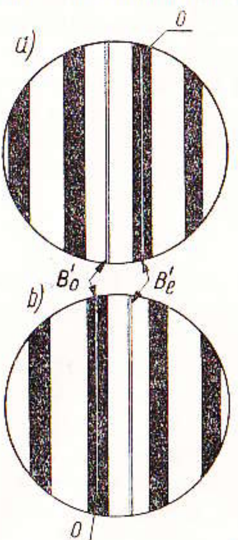


Fig. 20: Method of finding constant p' for a birefringent prism (No. 2) providing fringed interference field effect
a) Centre of dark zero interference fringe (0) when set for an extraordinary image B'_e; b) Centre of the same zero fringe (0) set for an ordinary image B'_o of a thin fibre or a fine scratch

to use an object slide with a thin scratch or fibre instead of a specimen and to apply an ×10 or ×20 objective. This scratch or fibre must be set parallelly with the interference finges. After having precisely focused the microscope it will be necessary to shift the birefringent prism transversely and to focus centre of the dark zero fringe (or any other one) first onto one and then onto other scratch image (Fig. 20) while reading from the micrometer screw thimbles amount of the birefringent prism displacement effected during this operation.

For approximate values of the constant p' applying to fringe prism (prism No. 2) refer to Table II.

3. DETERMINATION OF THE IMAGE SHEARING VALUE

The image shearing value r is to be known in relation to the micrascope object plane in order to enable measurement of the optical path difference gradient, tangential angle, refractive index and other quantities to be found by the differential interferometric methods (prism No. 1). This image shearing depends on the magnifying power of objective and must be found separately for each objective under the normal operating conditions.

The most simple way of finding this value is to employ a ×12 orthoscopic eyepiece (or micrometer eyepiece) and to measure the distance between images split for a very fine scratch or any other extremely small object which is to be always found on the object glass surface.

The image shearing value read then from an eyepiece scale is to be calculated next so as to get the shearing value related to the microscope object plane. The easiest way for this evaluation are microns. To do this, a micrometric slide — the so-called object micrometer — must be used to determine how many slide graduations fall to one scale interval of the micrometer eyepiece.

One graduation of the micrometric slide supplied with the microscope is 10 μm, the entire scale being 1 mm long. For theoretical values of the shearing effect for various objectives refer to Table II.

Chapter 5

MEASUREMENT OF OPTICAL PATH DIFFERENCE

1. FRINGED FIELD MEASUREMENT — BIREFRINGENT PRISM No. 2

This method appears to be especially suitable for examining fine fibres and narrow sections, separated cells and different oval objects not exceeding 32 μm in their diameter, as well as, thin layers and films.

In its most simple form, the measurement of an optical path difference consists in the determination of inter-fringe spacing h' within the microscopic field of view and in finding the amount of deviation d of interference fringes within the image of specimen being examined (Fig. 2).

By knowing these quantities and wavelength λ of the white light having been used one can easily find optical path difference Φ from the following formula:

$$\Phi = \frac{d}{h'} \cdot \lambda \qquad (15)$$

The inter-fringe spacing h' and deviation of fringes d can be measured by means of the ×12 measuring eyepiece, or by means of a special micrometer eyepiece available against specific buyer's request. This eyepiece is being used quite in the same manner as other microscope eyepieces. Prior to taking a measurement the eyepiece must be well focused to get a sharply contoured image of the micrometer scale. For this purpose, the focusing lens of said eyepiece has to be operated until a maximum sharpness of the scale image is produced.

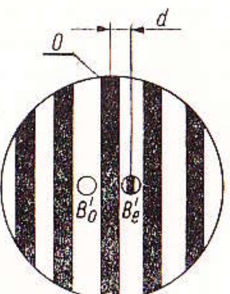


Fig. 21: Optical path difference as measured in the event of small oval objects seen in a fringed interference field

If a fibre, some narrow specimen or any other elongated section is to be examined, it will be preferable to position same by an angular 45° adjustment of the stage in relation to the direction of interference fringes (Fig. 2).

When applying the white light rays it is advisable to perform a measurement by using the dark zero fringe or the first colour fringe as reference. A convenient practice includes in this case reading of a double value d considering, at the same time, the amount of distance between centres of one and the same fringe in both the ordinary and

extraordinary images of the object under examination (Fig. 2). Such a procedure can be, however, adopted only when the object under examination is homogeneous as regards both its thickness and refractive index.

In the event of heterogeneous objects, the measurement of an optical path difference can be referred to a specified point only, and not to the object as a whole. The position of an observed specimen must then be such that the centre of deviated fringe chosen for the measurement could coincide with the required point of one or the other image.

The same effect can be achieved in a much more convenient way by shifting the birefringent prism transversely with the aid of micrometer screw control *15* (Fig. 16) while the specimen remains all the time unmoved. An identical procedure is recommended in the case of oval microobjects (Fig. 21).

Where extensile isotropic objects are involved, whose tranverse dimensions do exceed the image shearing value, the measurement of an optical path difference can be carried out no otherwise as only within the split image area limits. By rotating mechanical stage of the microscope one can produce shearing of images for any desirable boundary areas of the object under examination, like e.g. a cell. Since a maximum value of the image shearing for prism No. 2 providing a fringed field is 32 µm (×10 objective), the diameter of an area in which the optical path difference can be measured by rotation of the specimen will be 64 µm. See Fig. 22 for explanations.

In the event of thin layers or films, this method of measurement can be applied to adequately narrow strips, throws or grooves having an angular setting versus the direction of interference fringes (Figs. 23, 24, 25).

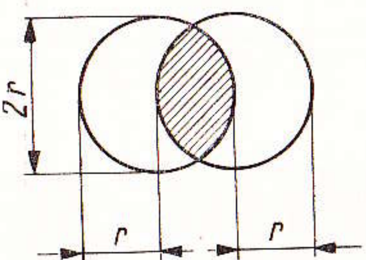
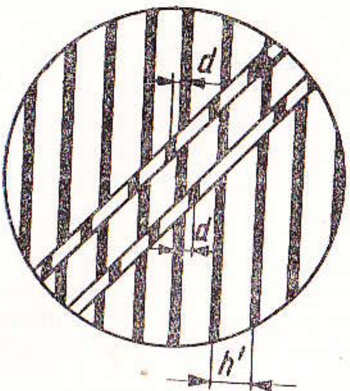


Fig. 22



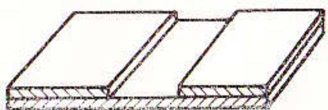
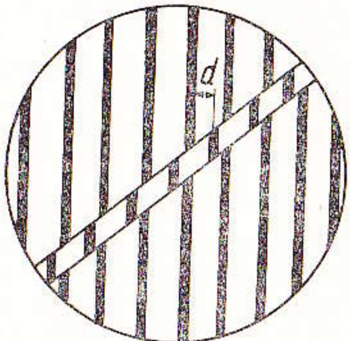


Fig. 23: Image of a sill in the fringed interference field



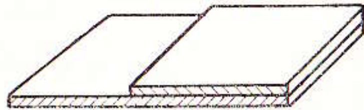
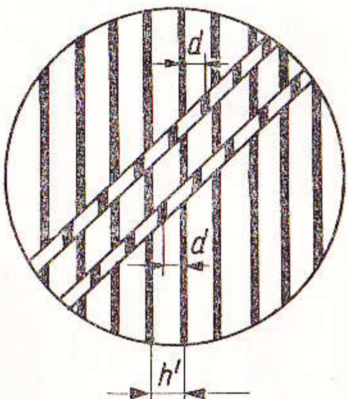


Fig. 24: Image of a step (vertical leap) in the fringed interference field



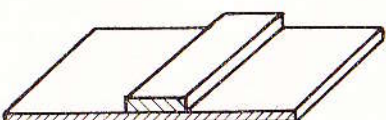


Fig. 25: Image of a groove in the fringed interference field

In the above manner one can measure optical path difference accurately to an order of $\frac{\lambda}{10}$. To achieve a higher degree of accuracy it will be necessary to take photographs of an interference image in order to improve precision with which maximum darkening of the interference fringes will be established by means of a microphotometer or densitometer. In this way, the accuracy in measuring optical path difference will rise to an order of magnitude of $\frac{\lambda}{20}$.

Another method of determining optical path difference in a fringed field includes measurement of the birefringent prism displacement $p\ (p=p_2-p_1)$ which becomes necessary to produce a maximum darkening of an identical fragment in both images being split with the zero interference fringe (Fig. 26).

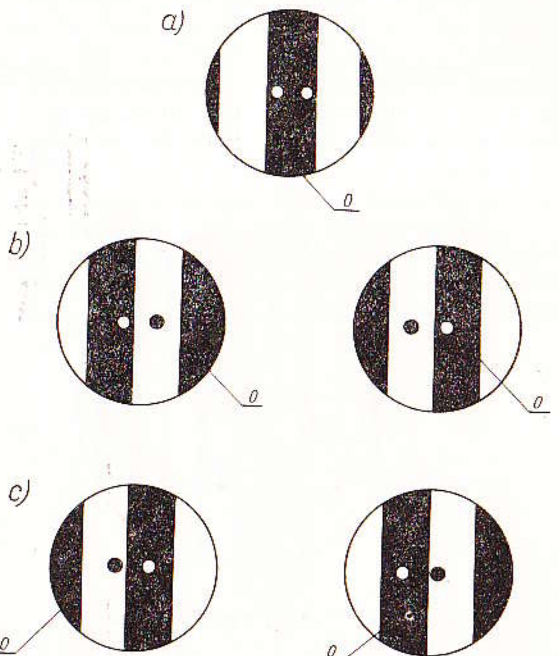


Fig. 26. Optical path difference measured in a fringed interference field with darkening of images and gauging of birefringent prism transversal shift

a) Dark zero fringe provided to the centre of image of the object under examination; *b*) Image of the object being investigated, darkened by a zero interference fringe (0); *c*) Another image darkened with the same zero interference fringe (0)

Oblong objects, like fine fibres, narrow strips and grooves, must during this operation remain parallel to the direction of interference fringes.

Optical path difference can be then expressed in the following mathematical way:

$$\Phi=\frac{1}{2}(p-p')\frac{\lambda}{h} \text{ for } n>n_1 \tag{16a}$$

$$\Phi=\frac{1}{2}(p+p')\frac{\lambda}{h} \text{ for } n<n_1 \ \&\ p>p' \tag{16b}$$

$$\Phi=\frac{1}{2}(p'-p)\frac{\lambda}{h} \text{ for } n<n_1 \ \&\ p<p' \tag{16c}$$

where:

n — refractive index of the object under examination;
n_1 — refractive index of the immersion medium;
λ — applied light wavelength;
p'— constant for the specified birefringent prism;
h — birefringent prism inter-fringe spacing.

The inter-fringe spacing h must not be confused with the inter-fringe spacing h' referred to above. The value h which may be defined as the actual inter-fringe spacing results from design features of a birefringent prism itself and is expressed by formula (1). For light wavelength being given this will be a constant figure dependent neither on the objective nor on the eyepiece power. The inter-fringe spacing h and constant p' for a white and monochromatic light of a wavelength $\lambda=590$ nm and $\lambda=546$ nm respectively can be had from Table II, or be found in a manner as specified above. Since the value of p in Table II has been given in microns, displacement p must be expressed in microns too.

Accuracy for this method is of an order of $\frac{\lambda}{30}$.

This method appears to be particularly suitable for the examination of isolated objects whose images are not entirely separated as yet.

2. UNIFORM COLOUR MEASUREMENTS INCLUDING HIGH IMAGE SHEARING EFFECT — BIREFRINGENT PRISM No. 3

A most universal method of measuring optical path difference with the aid of prisms No. 3, providing an uniform field of view with high image shearing, consists in a transverse displacement of the birefringent prism and in reducing colouration of one of the images having been split to that of the field of view. It is preferable to use for this

purpose a dark colour of the zero interference order. The measurement of optical path difference proceeds by stages so that first a zero position p_0 is found for the birefringent prism to produce maximum darkening of the microscopic field of view (Fig. 27a) and only thereafter the prism can be shifted in an appropriate direction to position p_1 where a maximum darkening of one of the images being split (Figs. 27 b or c) may be achieved. In accordance with the formulae (3) and (4) optical path difference Φ can be written as follows:

$$\Phi=(p_1-p_0)\frac{\lambda}{h} \qquad (17)$$

where:
λ — light wavelength applied;
h — birefringent prism inter-fringe spacing.

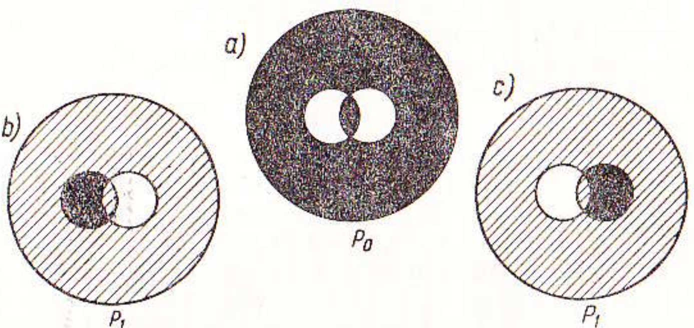


Fig. 27: Optical path difference measured by a method of darkening one of the split images of the object under observation in an uniform interference field

As a consequence, the measurement of Φ can be simply reduced to the micrometer screw thimble readings providing the amount p_1-p_0 by which the birefringent prism has been displaced.

The inter-fringe spacing h is established in the manner as described above, or it can be had from Table II if only measurements have been conducted in the white or monochromatic light with the wavelengths $\lambda=546$ nm or $\lambda=590$ nm.

Since the values of h in Table II have been presented in microns also displacement p_1-p_0 must be expressed in microns basing on the knowledge that an elementary division on the movable thimble is 10 µm and this on the fixed thimble 1000 µm.

Let us assume, for instance, that the measurement is conducted in a monochromatic light with a wavelength $\lambda=546$ nm. The position p_0 as read from the micrometer screw includes 4 divisions on the fixed thimble and 34 divisions on the movable thimble whereas position p_1

5 divisions on the fixed thimble and 96,5 divisions on movable thimble. By finding displacement of birefringent prism No. 3 in microns the p_1-p_0 difference will be=5965—4340 i.e. 1625. Consequently, the optical path difference

$$\Phi=(p_1-p_0)\frac{\lambda}{h}=\frac{1625}{830}\lambda$$

If both images of the object under examination remain entirely split, then it will be preferable to adopt a procedure in which the birefringent prism being moved will produce successive maximum darkenings

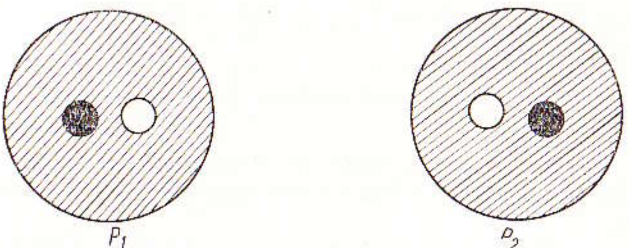


Fig. 28: Optical path difference measured by a method of darkening one and then also the other image of an object under observation in the uniform interference field

of one image and then the second image at one and the same point (Fig. 28). In this case, the optical path difference will read:

$$\Phi=\frac{1}{2}(p_2-p_1)\frac{\lambda}{h} \qquad (18)$$

where: p_2-p_1 is the difference between two positions of the birefringent prism, in which primarily the first and then also the second image become successively extinct to a maximum extent. This practice can also be recommended for the event when both images remain not completely separated (Fig. 27) and the object under examination is, nevertheless, uniform as to its thickness and refractive index and no need arises for the extinction of images at strictly one and the same points.

Should the optical path difference Φ for the object under examination be less than the light wavelength ($\Phi<\lambda$), it will be possible to adopt a procedure by which the birefringent prism can be set in such positions p_1 and p_2 in which the quality of one and then the second image remains the same as the field of view brightness (Fig. 29). In

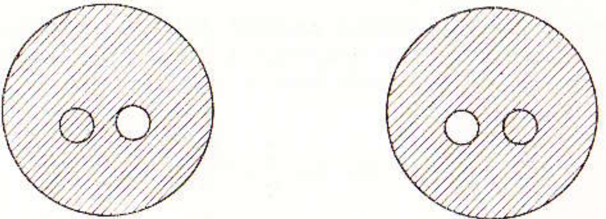


Fig. 29: Optical path difference measured in an uniform interference field by a method of extinction (compensation to suit brightness or colour of the background) applied to one and then also to the other image of the object under observation

these circumstances, the optical path difference Φ can be expressed by the following formula:

$$\Phi = (p_2 - p_1)\frac{\lambda}{h} \tag{19}$$

where: $p_2 - p_1$ represents a difference between birefringent prism positions in which first one and then also the second image become invisible.

If only one image appears in the microscopic field of view (e.g. when measuring throws), then by equalizing brightness of such an image with that of the field of view background a half value of the displacement $p_2 - p_1$ and the optical path difference Φ can be had from the formula:

$$\Phi = 2\,(p_1 - p_0)\frac{\lambda}{h} \tag{20}$$

where: p_0 means prism setting for the maximum darkened background and p_1 a setting at which both image and the background remain equally bright. The setting of prism for an equal brightness of the field of view background and image of the object under examination is more sensitive than a setting for maximum darkening, and for this reason the measurement by this method can be more accurate. It is possible, however, only when the object under examination yields an optical path difference not exceeding 1 λ.

Since the maximum image shearing value r for prism No. 3 is 6.5 μm, the diameter of an area within which optical path difference can be measured by the methods referred to above is 13 μm (angular movement of the stage) for each point of the object under consideration. Hence, this diameter is smaller than in case fo fringed prism No. 2. However, it is the accuracy of measurement which is much better when establishing optical path difference by means of prism No. 3 and this accuracy can in some instances be as high as $\frac{08}{\gamma}$.

When fine lamellae, layers or films are subject to investigation, the optical path difference can be measured by means of prisms No. 3 on adequately narrow strips, throws and grooves with their sharp edges disposed more or less parallelly towards refracting edge of the birefringent prism. Fine fibres and oblong objects must also be set up in a similar manner. Fig. 55 shows an example on how to measure optical path difference for the red blood cells (compare Figs. 27a, b, c).

Methods described in the foregoing exhibit also some disadvantages. In the first method with successive darkening (maximum darkening) of first one and then also the second split image, visual examination and estimation of the maximum darkening effect remains somewhat subjective. The human eye appears namely not sensitive enough to perceive the minimum brightness (i.e. maximum darkening) of the field of view or its fragment. Another method consisting in balancing the brightness of the first and the second image to match it with that of the field of view background can find use only in the event of very fine homogeneous objects.

In biological investigations where heterogeneous specimens undergo the process of investigation only the first method appears to be suitable.

On the other hand, the human eye very well perceives any difference in the brightness of two adjacent fields. This feature has been utilized in a special half-shade eyepiece attachment which provides means for a more accurate measurement of the optical path differences than it would be feasible by the method of a maximum darkening of images split. The main component of this eyepiece includes a half-wave plate located in the eyepiece object plane. By moving the stage, the investigated object can be positioned so that one edge of the half-wave plate strip would form a line of separation for the two parts of images of this object having been split.

The measurement of optical path difference includes transverse shifting of prism W_1 and ensuring to it two such locations at which brinhtness of the first and then also of the second split image would have been identical within the half-wave space and outside it. Both locations p_1 and p_2 of the prism are read to calculate optical path difference from formula (18). The half-shaded eyepiece attachment has been designed so that both observation of the interference image and objective exit lens have been made possible at the same time. For this purpose a negative engageable lens is used. In the engaged position of this lens, the attachment provides a possibility for interference image observations with simultaneous equalization of the image bringhtness. The use of microscope attachment with a half-shaded eyepiece renders that optical path difference measurements can be made to an order of accuracy equal to $\lambda/500$.

NOTE: Whenever a half-shaded eyepiece finds use, it will be necessary to disengage analyser A (Fig. 1). The function of this analyser will be performed by another one provided in the half-shaded eyepiece.

3. UNIFORM DIFFERENTIAL FIELD MEASUREMENTS — — BIREFRINGENT PRISM No. 1

As known, the optical path difference Ψ between the ordinary and extraordinary waves in a differential interference image does not expressly reflect optical path difference Φ within the object under examination but only the gradient of this difference towards the point at which interference waves become split (Fig. 8).

An optical path difference Ψ within the image cannot in such a case be identified with that caused by object under investigation. Optical path difference within a differential image, Ψ, is also easiest to be measured under an uniform dark colour of the zero interference order. With the birefringent prism set to an uniformly dark field of view (p_o zero position of prism) there will nearly almost appear such a point (or more points) within the investigated object image (of dimensions exceeding the shearing value r) where the observed colour will remain the same as that of the field of view background (see Fig. 47). At these points, fronts of the interference waves adhere each other (are tangent) and the shift reaches a zero value. If Ψ is to be measured at any other point of the object being examined, the birefringent prism will have to be shifted to a position p_1 where a maximum darkening is the outcome.

At this point, optical path difference can be expressed by the following formula:

$$\Psi=(p_1-p_0)\frac{\lambda}{h} \qquad (21)$$

Consequently, the process of Ψ measurement is reduced, as formerly, to the reading of prism shift p_1 minus p_o from the micrometer screw indications. Interfringe spacing h remains to be determined similarly to the previously described procedure.

Having measured optical path difference within a differential image area one can also find gradient of the differential path difference in an object (formula 12), tangential angle (formula 13), curvature (formula 14), index of light refraction (formula 15) and several other data.

Under favourable conditions of observation, this method ensures an accuracy of measurement for the optical path difference Ψ of an order of $\lambda/250$.

Chapter 6

MEASUREMENT OF REFRACTIVE INDEX AND THICKNESS

1. ESTIMATION OF REFRACTIVE INDEX VERSUS THE SURROUNDING MEDIUM

Let us suppose that object B under examination (Fig. 30), having an unknown refractive index n, rests in an immersion medium of a known refractive index n_1.

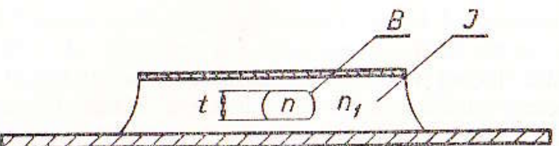


Fig. 30

If we now want to find refractive index n it will be necessary to state primarily whether it exceeds or not refractive index n_1 of the immersion medium J. In a phase-contrast microscopy this problem can be solved immediately, but in an interference microscopy the answer is not as easy and requires a certain analysis of the change in interference colours or in the direction of interference fringe deviation. Such an analysis will be easiest to be carried out by the uniformly dark colour or dark zero fringe method.

1.1. Differential Method (Prism No. 1)

As mentioned already before, a specific feature of this method is that within the image of a variable thickness object there will nearly always occur such a point (or more points) where the colour will be identical with that of the background (see e.g. Figs. 47 and 50).

This will be particularly distinct whenever the birefringent prism has been set for the colour of the microscopic field-of-view background. With the birefringent prism being moved in the transverse direction, the dark colour in the observed image will also be subject to a certain amount of displacement. The direction of this displacement will be consistent or not with that of the prism movement, and this will be dependent on whether refractive index n of the object under investigation will be higher or lower than that of the surrounding medium, n_1.

If the direction of dark colour displacement in the image under observation remains consistent with that of the birefringent prism shift (the prism being initially set for the dark field-of-view background), refractive index n of the object will be higher than that of the sur-