STUDIES ON DEPOLARIZATION OF LIGHT AT MICROSCOPE LENS SURFACES

II. The Simultaneous Realization of High Resolution and High Sensitivity With the Polarizing Microscope*

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I. The Value and Limitation of Polarization Microscopy in Biology:

The uniqueness of polarization microscopy lies in the use of light beams with controlled states of polarization. With such beams, optical properties such as birefringence, dichroism, and optical activity are detectable, and optical constants are determinable in an exceedingly small sample volume. Since birefringence and dichroism generally reflect the arrangement or properties of material the dimensions of which lie below the limit of resolution of the light microscope, the polarizing microscope can in principle be used for studying the optically unresolvable "submicroscopic structures" of cells and tissues. Such studies can be made directly on living cells and the submicroscopic events followed as the cells undergo physiological activities. In contrast, the electron microscope gives a vastly improved resolving power and enables a direct visualization of the submicroscopic structures, but only after their fixation and desiccation. The two instruments, therefore, are complementary to one another, and together they make up for each other's shortcomings. Indeed Sjöstrand (9, p. 511) has commented on these aspects of electron microscopy, concluding that " . . . the polarized light microscope should be the standard equipment of the electron microscopists. But it has to be used on living cells and it has to be equipped for analyzing very weak birefringence. . . . "

Although potentially endowed with such desirable and unique attributes, the polarizing microscope has not yet been used very extensively for studying living cells. This is not due to the absence of a submicroscopic organization in cells, for such has been amply demonstrated by recent electron microscope

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studies, nor to the lack of birefringence and dichroism in the cytoplasmic and nuclear elements, for W. J. Schmidt in his celebrated monograph (8) has long ago demonstrated their wide distribution.

The optical problems that confront the biologist who wishes to apply a polarizing microscope to the study of live cellular structures are twofold:

1. The method must be sensitive enough to produce perceptible contrast in objects whose maximum retardation (strength of birefringence × thickness) may lie well below a hundredth of a wave length. In general, a sensitivity of an angstrom unit or less is required.

2. The retardation must be detectable and rapidly measurable in regions or in structures whose dimensions may lie close to the limit of resolution of the light microscope.

Methods for obtaining maximum sensitivity for detecting weak birefringence have been described by Schmidt (7), Swann and Mitchison (10), and Inoué and Dan (5) (also see Wright 11). Methods for measurement of low retardation of small objects have been described by Swann and Mitchison (10), and by Inoué (3). In all of these references, strain birefringence and other sources of stray light are removed to gain maximum extinction; a very bright light source is used to aid the sensitivity of the eye and to reduce the exposure time for photographing the object which may constantly be changing; and a mica compensator is used to improve contrast and field brightness.

When these precautions are taken, the sensitivity and resolution of the polarizing microscope ultimately become limited by depolarization of light at the lens and slide surfaces. The depolarization, whose nature is explained below, results in stray light whose intensity increases approximately exponentially as the numerical aperture is increased. The stray light reduces contrast and drowns the image of weakly birefringent objects. This necessitates the use of low numerical aperture objectives and/or considerable stopping down of the condenser diaphragm. In either case the resolving power is lowered. Indeed *high sensitivity and high resolution have appeared to be mutually exclusive qualities of polarizing microscopes* (1).

II. The Polarization Rectifier:

In a conventional polarizing microscope equipped with high extinction polarizers and in excellent adjustment, the extinction diminishes rapidly as the numerical aperture is increased. Inoué (4) has earlier shown that the increased amount of stray light at higher numerical apertures measured photoelectrically agrees closely with the amount of light calculated to enter the system by depolarization, assumed to occur solely by the rotation of the plane of polarization at lens and slide surfaces. At these surfaces, the component polarized in the plane of incidence suffers less reflection loss than the component polarized perpendicular to it, and the plane of polarization of the transmitted light is

rotated towards the plane of incidence. Only when the plane of incidence lies parallel or perpendicular to the plane of polarization is the beam transmitted without rotation. When the polarizer and analyzer are crossed, a dark polarization cross therefore results, which can be seen at the back aperture of the objective. The rotation can be reduced somewhat by low reflection coating of the glass surfaces and by the use of oil immersion lenses, but even then it continues seriously to limit the extinction and the polarization cross persists.

When the condenser and objective lenses and the specimen slide are free from (lateral) strain birefringence the polarization cross is symmetrical and complete (Fig. 1 *a*). As the polarizer is rotated, the cross opens up into two dark V's which move out symmetrically to the edge of the aperture (Fig. 1 *b*). Loss of contrast in the V's is generally an indication of (radial) strain birefringence or elliptical polarization due to the coating on the lenses. Rotation of the polarizer in the opposite direction results in V's in the two other quadrants. These V's represent regions of the objective aperture through which rays suffering equal degrees of rotation have traveled. The degree of rotation thus varies with aperture and azimuth and the sense of rotation is reversed in adjacent quadrants.

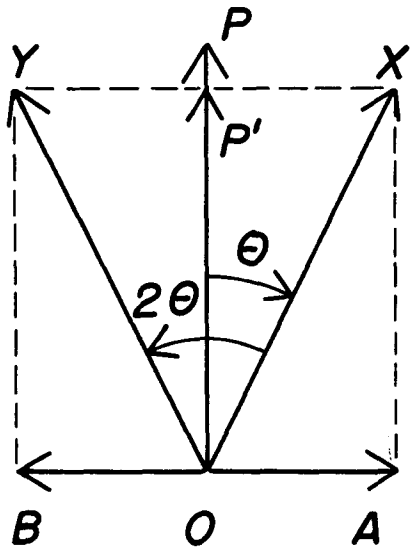
For any particular ray the sense of rotation is the same in the condenser, objective, and specimen slide, and cannot be reduced by the simple expedient of adding lenses to either. The rotation can be reduced or even removed, however, by a second spherical lens system if the rotatory sense of the error introduced by the two systems can be made opposite. This can be accomplished by inserting a half-wave plate[1] between the two optical systems with its axis parallel to the polarizer (Text-fig. 1). Those rays for which the plane of polarization is rotated before entering the half-wave plate will emerge from it with an error of the same magnitude but opposite sense. This effect is found irrespective of the magnitude and sense of the original error.

In principle, such a half-wave plate might be placed between the condenser and the objective, letting the rotation of the one compensate for that of the other. This is not feasible because no available half-wave plate has uniform properties at all the angles of incidence found in this region. The half-wave plate must lie in a path where the beams are nearly parallel. It could, for example, be inserted between two entirely separate but duplicate optical systems, but this would be awkward and expensive. A simpler and more practical system we have devised is based on the following reasoning.

It is found that the rotation imposed on each and all of the rays by the regular condenser-slide-objective system increases proportionately when coverglasses are added (without oil immersion) between the condenser and objective lenses (Inoué, unpublished data). By choosing a proper number of coverglasses

[1] For the explanation of rotation of the plane of polarization by a half-wave plate, see *e.g.* references 2, 6.

or by altering their refractive indices, the rotation of each ray can be exactly doubled. If now the coverglasses are transferred below the condenser and given a suitable curvature, the resulting meniscus would continue to have the same effect on rotation, since the radius of each zone at the front focal plane of the condenser is proportional to the sine of the angle of the corresponding ray in the object plane. If a half-wave plate is now inserted between the meniscus and the condenser, it will reverse the rotation of each ray, and rotation at the meniscus will cancel out the rotation introduced by the condenser, objective lens, and



TEXT-FIG. 1. Effect of a half-wave plate on a ray whose plane of polarization OX was deviated by an angle θ away from the polarizer axis OP. The half-wave plate, whose axis is parallel to OP separates $\overrightarrow{OX}$ into $\overrightarrow{OA}$ and $\overrightarrow{OP'}$. Then after a half-wave retardation, $\overrightarrow{OB}$ and $\overrightarrow{OP'}$ are combined to give $\overrightarrow{OY}$. The plane of polarization OX is thus rotated -2θ to OY and the deviation is effectively reversed.

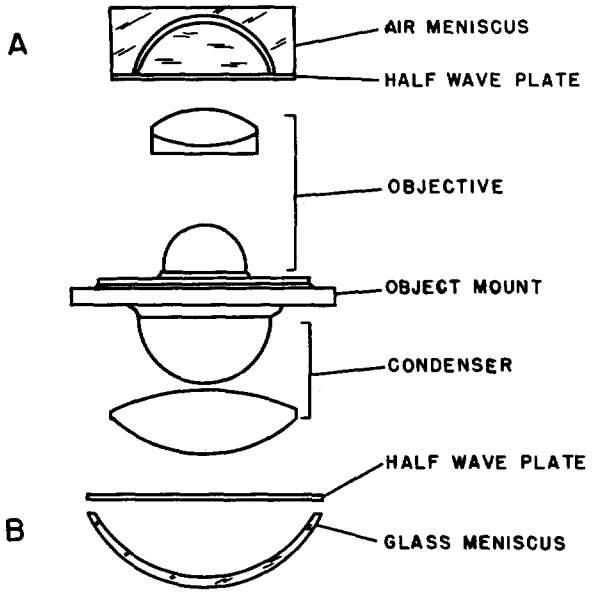
the specimen slide. Such a combination of a half-wave plate and a meniscus we have named the *polarization rectifier.*

The rectifier can be adjusted by moving it along the optical axis of the microscope until the rotation at each point of the meniscus is precisely matched with the rotation at each point of the lens system. This adjustment is possible because the gradually narrowing cone of rays approaching the center of the field stop (at the light source) passes a more or less steeply curved region of the meniscus.

The rectifier can be placed under the condenser (Text-fig. 2 B), above the objective (Text-fig. 2 A), or at both places. The first method is adequate, when only high extinction is required, or when there is no marked deviation of

the beam by the object. Whenever image quality is emphasized, however, the last method is preferred, since otherwise the beams diffracted and deviated by the object will be incorrectly rectified in the objective. The result is that some objects will become visible merely because of their small size, whether birefringent or not.

The rectifying rotation can be provided either by a deeply curved zero-power lass shell (Text-fig. 2 B) or a thin air bell produced between a pair of convex



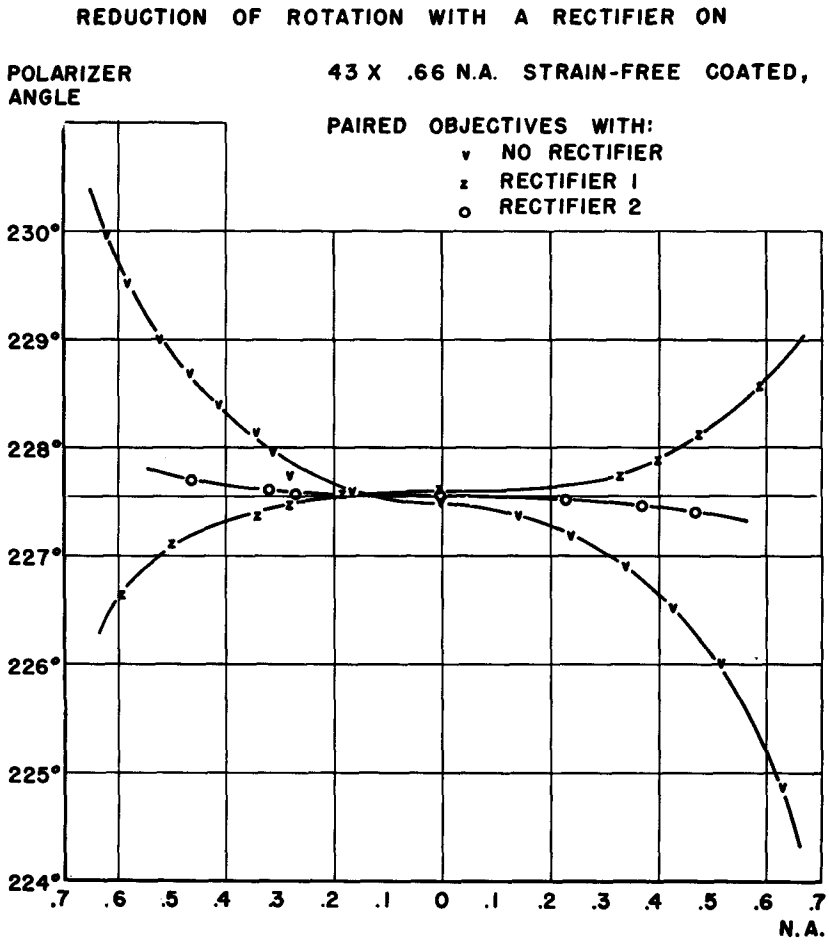
TEXT-FIG. 2. Arrangement of polarization rectifiers. Rectifiers are placed above the objective (*A*), below the condenser (*B*), or at both *A* and *B*. The glass or air meniscus introduces additional rotation to each beam of light which equals the amount introduced at the lenses and the specimen slide. The sense of rotation is, however, reversed by the half-wave plate so rectification is achieved over the whole aperture. All elements must be free from strain.

and concave glass surfaces (Text-fig. 2 A). In practice, it may also be possible to place the half-wave plate within the objective or the condenser, adjusting the amount of rotation of some or all of the surfaces by low or high reflection coating and letting part of the elements provide rectification for the other elements.

It should be remarked here that the half-wave plate of the rectifier oriented properly changes the sign, but not the magnitude of the ellipticity of the light beam. The rectifier, therefore, does not affect the measurement of the amount of retardation but, since it effectively changes the two axes of the ellipse, a compensator placed beyond a single half-wave plate acts as though it were oriented in the wrong quadrants.

Although the rectifier is best used with monochromatic light, it has also been

used with reasonable success in white light. Further improvements can be expected by using two identical half-wave plates for each rectifier with their



TEXT-FIG. 3. Reduction of rotation by the use of rectifiers. Rectifier 1 is weak. The rotation is reversed but rectification is inadequate. Rectifier 2 has the proper curvature. It is placed too close to the condenser and shows a slight overrectification. The rotation measurements were made in the back aperture of an objective along axes at 45° to the axis of the polarizer.

axes mutually crossed.[2] Placed on each side of the meniscus, these wave plates would restore the original plane of polarization and certain errors in the half-wave plates would be cancelled out. So long as the two plates are identical,

[2] This arrangement was suggested by Dr. Stephen M. MacNeille of the American Optical Company.

greater deviation from half-wave conditions could be tolerated, and the influence of oblique beams or of "whiter" light should be less severe.

III. The Polarizing Microscope After Rectification:

The amount of rotation in a pair of coated objectives, before introduction of a rectifier, is shown in Text-fig. 3, curve *v*, as a function of numerical aperture. With a slightly overcorrecting rectifier, the rotation in the same objectives was reduced to curve *o* in the same figure. For comparison the effect of an undercorrecting rectifier is shown in curve *z*. In this and all of the following examples, we chose two identical objectives with minimum strain birefringence and used one of the two as a condenser. The special microscope on which these lenses were tested will be described elsewhere. In general, the maximum rotation of high numerical aperture objectives was reduced from 3 to 6 degrees to a few tenths of a degree by rectification. The exact minimum value of rotation could not be determined in many cases because of interference from a small residual strain birefringence.

The back aperture of a pair of 1.25 N.A. coated oil immersion objectives equipped with rectifiers is shown in Fig. 1 *c*. Compared to the same pair without rectifiers (Fig. 1 *a*), the extinction at high numerical apertures is greatly improved and the back aperture is uniformly dark. The original extinction factor of this pair of lenses was 600, whereas with rectification it rose to 13,000. Together with an improvement of extinction, spurious light is dramatically removed from the image as the rotations of the oblique rays are diminished. The theory and result of this effect will be described elsewhere.

The corresponding improvement of extinction and contrast in the image plane are illustrated in Figs. 2 and 3. Fig. 4 shows a portion of a human oral epithelial cell photographed in green light (Hg 546 mμ) with a 1.25 N.A. rectified objective and an identical condenser at full numerical aperture. The resolution is 0.2 micron or slightly better, while the retardation of the birefringent ridges in the picture ranges between a few angstrom units to a few millimicra. As illustrated, the sensitivity, resolution, contrast, and general quality of the image with rectified lenses are all excellent. Although the implications of the two sets of images are dissimilar, comparison with the phase contrast image also at N.A. 1.25 (Fig. 5) points to the effectiveness of the rectified polarization optical system as a contrast yielding device.

The introduction of the rectifier has overcome what appeared to be an inherent limitation to the extinction and resolution of the polarizing microscope. Both for orthoscopic and conoscopic applications, the sensitivity for detecting low birefringence and the precision in determining optical constants at high numerical aperture are vastly improved. Among other applications, the rectifier now makes possible accurate measurements of weak birefringence and dichroism in cell regions too minute to be resolved with polarizing microscopes in the past. The development and change of such anisotropic properties can be followed

directly on living cells undergoing mitosis, cell division, muscle contraction, etc. By correlating such studies with biochemical and electron microscope findings on cell fractions or on sections of fixed cells, we may hope to obtain a more integrated picture of the submicroscopic events underlying various physiological activities and pathological processes in the living organism.

The stand for the high sensitivity polarizing microscope for which this rectifying device has been prepared was built by one of the authors (S. I.) during his stay in Seattle with aid from the Biological and Medical Fund of the State of Washington. We are very grateful to the University of Washington and Dr. H. Stanley Bennett of the Department of Anatomy for making the microscope available for this work.

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EXPLANATION OF PLATES

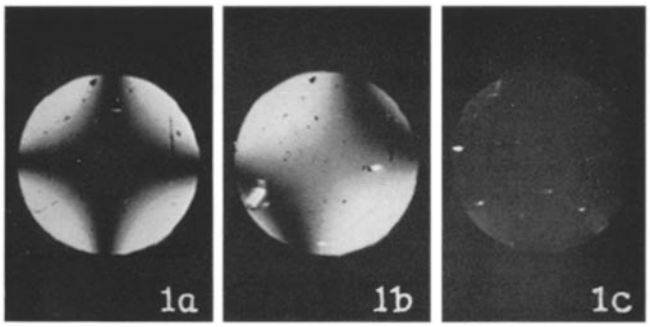
PLATE 262

FIG. 1. Appearance of the back aperture of a 97× 1.25 N.A. strain-free coated objective with and without rectifiers. The condenser, which is identical with the objective, is used at full aperture. Collimated mercury green light (546 mμ) used for illumination.

FIG. 1 *a*. Crossed polarizers, no rectifier.

FIG. 1 *b*. Polarizer turned 2°, no rectifier.

FIG. 1 *c*. Crossed polarizers, with rectifiers in both condenser and objective. Photographs *a*, *b*, and *c* were given identical exposures.

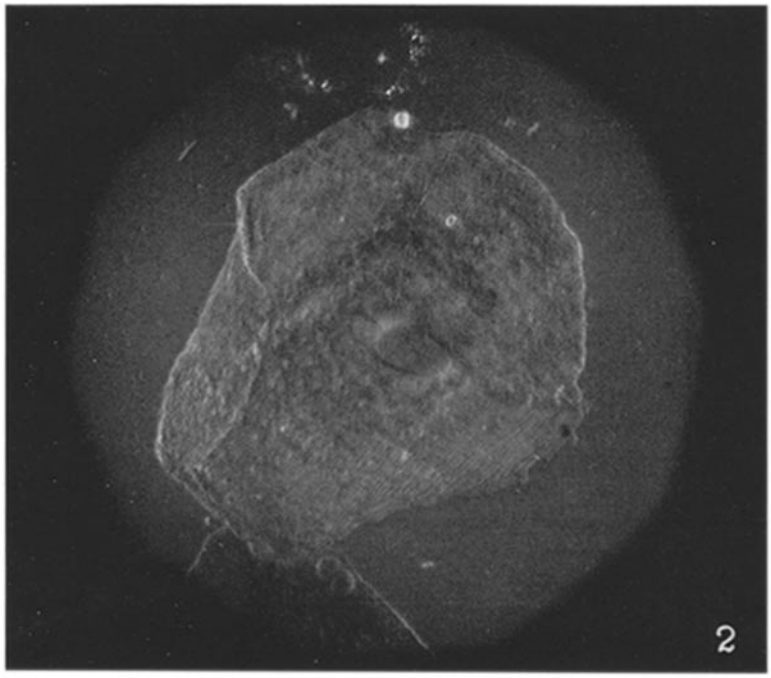


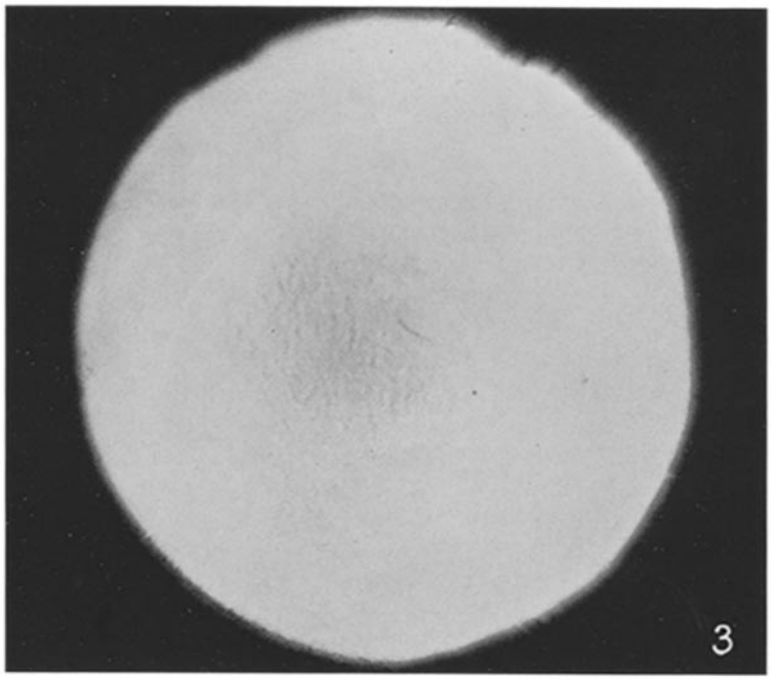
(Inoué and Hyde: Studies on the polarizing microscope. II)

PLATE 263

FIG. 2. Photograph of an epithelial cell from a human mouth taken with a 43× 0.85 N.A. strain-free coated objective and a 43× 0.63 N.A. strain-free non-coated condenser equipped with a rectifier. Polarizers crossed, 1 mμ background retardation. (× *ca.* 680).

FIG. 3. Identical to Fig. 2 but without a rectifier. Notice the brighter background and the total lack of detail and contrast in the image.



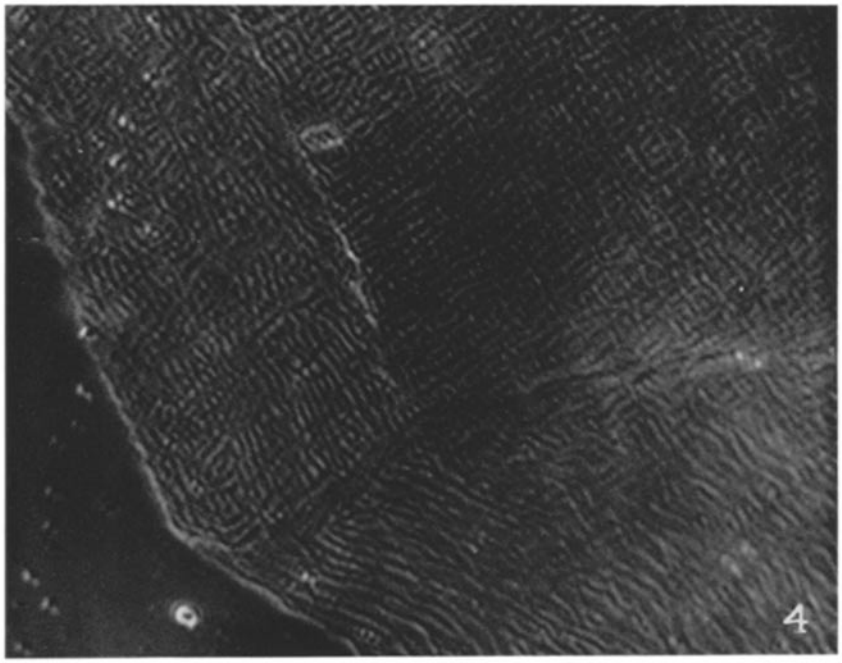


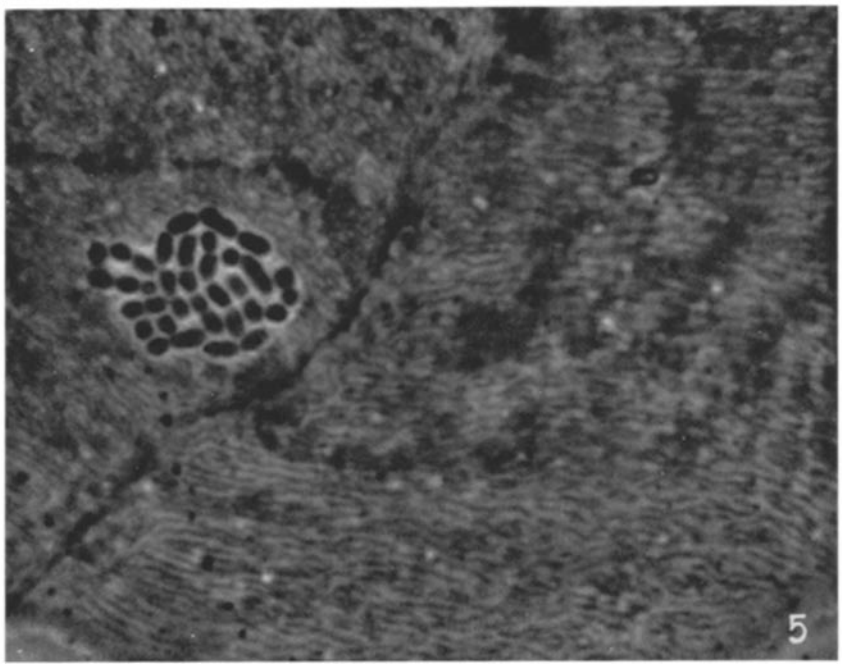
(Inoué and Hyde: Studies on the polarizing microscope. II)

PLATE 264

FIG. 4. A high magnification image (× *ca.* 3600) taken with the rectified optics showing birefringent surface structures (dots and ridges) of a portion of a cell similar to the one shown in Figs. 2 and 3. 97× 1.25 N.A. strain-free coated objective and 97× 1.25 N.A. strain-free coated condenser are both used at full aperture and separately rectified. Polarizers crossed, background retardation *ca.* 5 mμ.

FIG. 5. Phase contrast image (× *ca.* 3000) showing the surface structure of an adjacent portion of the same cell shown in Fig. 4. 97× 1.25 N.A. phase contrast objective with annulus diaphragm in the condenser. The resolution is comparable to the polarization optical image in Fig. 4, but contrast is produced by entirely different principles.





(Inoué and Hyde: Studies on the polarizing microscope. II)