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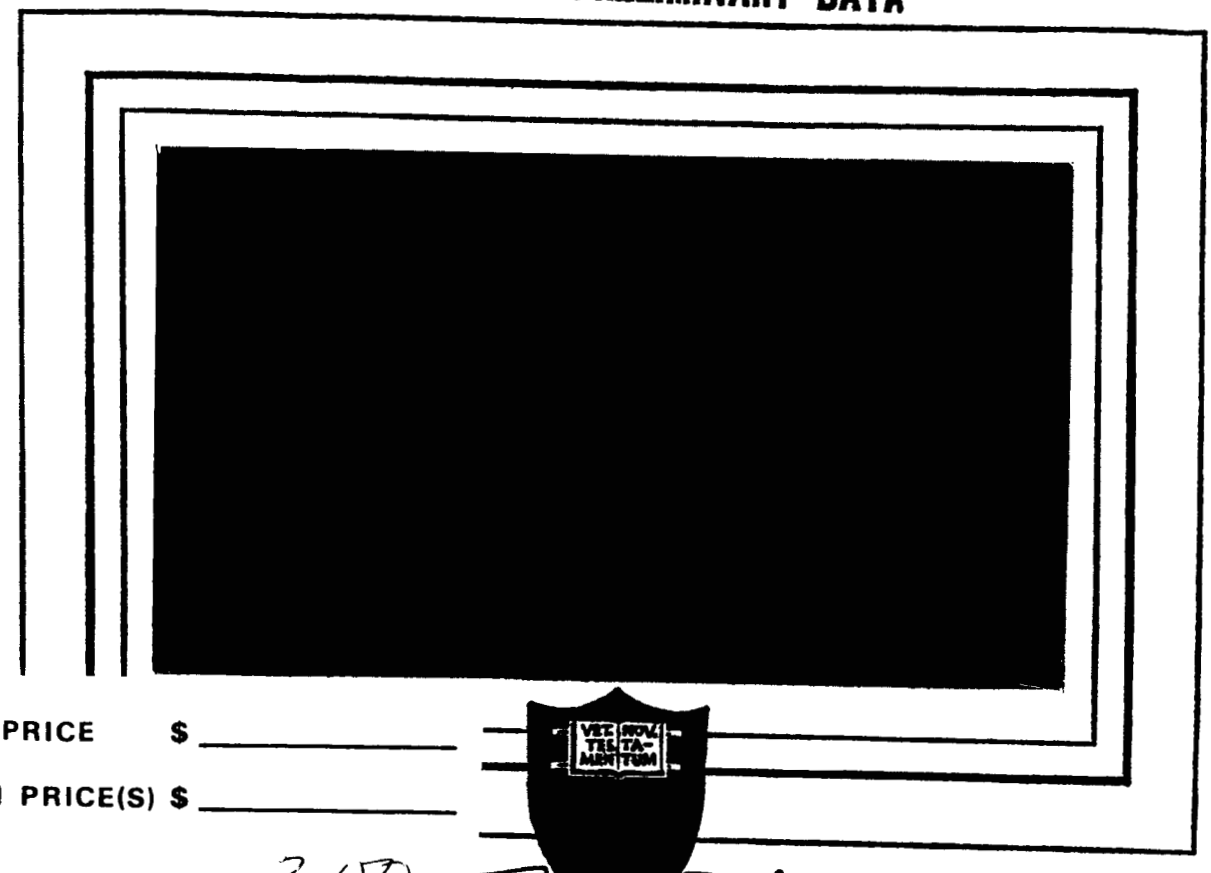

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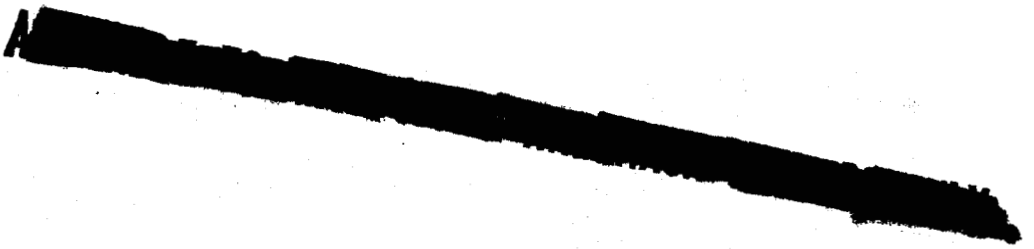

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Part I Image Contrast and Phase Modulated Light Methods in Polarization and Interference Microscopy [1]

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IMAGE CONTRAST AND PHASE-MODULATED LIGHT METHODS IN POLARIZATION AND INTERFERENCE MICROSCOPY

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CONTENTS

1. PHASE RETARDATION MEASUREMENTS IN POLARIZATION AND INTERFERENCE MICROSCOPY

Both polarizing and interference microscopes detect and measure changes or differences in the velocity of light propagated within minute objects. Both instruments accomplish this by measuring the retardation or advancement of one train of light waves relative to another emanating from the same source. Both types of measurements also require (except in special circumstances, see Section 2,D.) the determination of the thickness of the object. These measurements in the two kinds of microscopes present similar problems even though the optical properties detected are in the two cases different. This article will compare the theory and practice of measurements of phase retardation by two entirely different methods; first by the generation of image contrast through interference as the basis for photometric measurements and second, the electronic detection of phase retardations by phase-modulation methods employing "lock-in amplifier" techniques.

Light emitted from a given monochromatic source is considered according to physical optical theory as trains of waves vibrating at a characteristic frequency (ν) which is invariant. The velocity (V) and wavelength (λ) of light both change in media of different refractive index. The relationship between frequency, velocity, and wavelength is

$$\nu = V/\lambda \qquad \text{.....(1)}$$

For light of a given frequency, the refractive index of an

isotropic medium is the ratio of the velocity of light in a vacuum to the velocity of light in that medium:

$$n = \frac{V \text{ (vacuum)}}{V \text{ (medium)}} \quad \ldots\ldots\ldots\ldots\ldots (2)$$

Anisotropic or birefringent materials are crystalline and transmit, at different velocities, light waves which vibrate parallel to their crystalline axes.

Birefringence (B) is usually defined as the difference in refractive indices (n_1, n_2) of material for light vibrating parallel to its crystalline axes. However, birefringence is usually measured with monochromatic light as the retardation (Γ) in linear units (millimicrons or Ångstrom units) per identical unit of thickness, (t).

$$B = n_1 - n_2 = \frac{\Gamma}{t} \quad \ldots\ldots\ldots\ldots\ldots (3)$$

The retardation of one wave of monochromatic light relative to another can be expressed not only in linear units (Γ), but as fractions of a wavelength (e.g. $\lambda/4$ for quarter wave), or as a phase angle in degrees (δ) or in radians (Δ).

$$\frac{\Gamma}{\lambda} = \frac{\delta}{360} = \frac{\Delta}{2\pi} \quad \ldots\ldots\ldots\ldots\ldots (4)$$

(For general references, see Jenkins and White (1957), Ditchburn (1963) and Shurcliff (1962).)

The measurement of birefringence requires that both the phase retardation and the thickness of the specimen be measured. The polarizing microscope is used to find and identify the crystalline axes of the object by turning it on a revolving stage in a field illuminated with linearly polarized light. The simplest conditions for measuring the phase retardation are obtained by rotating the object 45° from

the position of minimum brightness in linearly polarized light. At the 45° position the brightness of the object should be maximal. The introduction of a phase retardation from a compensator of the proper range equal and opposite to that of the specimen will bring its brightness to a minimum, called the extinction point. Since the slow axis of the compensator is marked (usually with the symbol γ), the slow axis of the specimen is then known and the sign of its birefringence noted according to the convention that positively birefringent objects transmit light more slowly parallel to their long axis (see Bennett, 1950, for details).

When the specimen is oriented at 45° to the incident linearly polarized light, the latter is resolved into two mutually perpendicular components of an elliptically polarized wave. The phase retardation of one of these components relative to the other can be measured with any of several kinds of compensators, such as the tilting calcite plates, e.g. of Ehringhaus (1931), a sliding bi-wedge of the Soleil-Babinet type, a rotatable mica plate of the Köhler (1921) type, or by a fixed quarter-wave plate followed by a rotatable analyzer (the de Sénarmont method 1840, 1847). (See Jerrard, 1948, for a discussion of the relative merits of compensators.) With the Ehringhaus and Soleil-Babinet compensators (the so-called "fixed azimuth type") the phase of the two perpendicular waves is shifted without resolving them into new components at different angles. The mica compensator is more complicated; it is

biaxial and phase shifts are introduced by varying the angle of its crystalline axes to those of the object to be analyzed. In the de Sénarmont method, the fixed quarter-wave plate may be considered either to resolve the phase-retarded 45° linear components of the elliptically polarized light into counter-rotating, circularly polarized waves which interfere at an analyzer angle characteristic of the original phase retardation between them or to convert a phase retardation into an equivalent optical rotation. The advantages of the de Sénarmont method are that 1) the range is one wavelength (for 180° rotation of the analyzer), and 2) the scale is linear. These and other compensators vary both in range, and in the accuracy with which their scales may be read. The theory and practice of compensator use are discussed at length in several important publications which should be consulted for details. (Jerrard, 1948; Bennett, 1950; Gahm, 1963; Hallimond, 1953; Hartshorn and Stuart 1951; Inoué, 1951; Inoué and Koester, 1959.)

The double-beam interference microscope measures principally the retardation of light waves transmitted through isotropic specimens relative to those waves transmitted through their surround. This retardation, or optical path difference, is proportional to the thickness of the specimen and to the difference in refractive index from that of the surround.

$$O.P.D. = t(n_o - n_m) \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

One type of interference microscope now manufactured by

the E. Leitz Company, the Mach-Zehnder type (Horn, 1958), utilizes unpolarized light which is divided by beam-splitting prisms into paths traversing microscopes of matched optical paths, only one of which contains the specimen. Light from the two microscopes is recombined by a second set of prisms to produce interference color contrast in white light or brightness contrast in monochromatic light. In this type of interference microscope the mean refractive index is measured unless polarizing filters are used to detect birefringence. Optical path differences or phase retardations are measured by isotropic wedge compensators moved by screw mechanisms.

A second type of instrument (Dyson, 1950) now manufactured by Vickers Instruments, Ltd., accomplishes a similar result by half-silvered mirror beam splitters within a single microscope. In general, it is troublesome and inconvenient to set up, and it may not be used to detect birefringence.

A third type is the polarizing interference microscope (Lebedeff, 1930), different versions of which are available from various optical companies. For example, Vickers (formerly Baker) and the American Optical Company, manufacture the device patented by Smith in 1947 and 1950 (see Smith, 1955, for references); the Zeiss Company has produced a modified version based on the same principle (Piller, 1962, Gahm, 1963). In these instruments the phase retardations caused by total refraction (and to some extent influenced by birefringence) are identical from the physical

standpoint, to those caused by birefringent specimens oriented at 45 degrees to the plane of polarization in the polarizing microscope. For this reason, identical compensation methods may be used to measure these phase retardations in the two cases.

The feature of polarizing interference microscopes which determines that the phase retardations observed are due to total refraction and not to birefringence is a pair of birefringent plates situated below and above the specimen plane. The first of these resolves each incident linearly polarized ray into two mutually perpendicular linearly polarized rays vibrating at 45 degrees to the incident plane of polarization and traversing different parts of the specimen plane. One of these rays constitutes part of the "measuring beam", while the other is part of the "reference beam". The object retards (or advances) the former but not the latter. When the two are recombined by the second birefringent beam-splitting plate, the phase retardation introduced by refraction in the specimen is detected as the phase retardation of one of the linear components of the elliptically polarized light. Since the measuring beam is linearly polarized, the retardation which is measured will depend, to some extent, on whether the specimen is birefringent, and if so, which of its crystalline axes is parallel to the plane of polarization in the measuring beam. Thus the polarizing interference microscope detects mean refractive index. However, double refraction or birefringence is also detectable, as a difference in

phase retardation as the specimen is rotated in the measuring beam. The sensitivity for birefringence, however, is far lower than with the polarizing microscope. (cf. Bruce and Thornton, 1957; Gahm, 1962, 1963.)

A. IMAGE CONTRAST METHODS AND THEIR LIMITATIONS

Phase retardation measurements in both interference and polarization microscopes generally depend on some kind of photometry in the image plane. The generation of contrast for such measurements is based on constructive and destructive interference of light waves differentially retarded by the specimen. In both the polarization and polarizing interference microscopes, the maximum contrast due to light interference is limited by the extinction factor, F, (also called the contrast ratio), which is the ratio of maximum and minimum intensities at peak constructive and destructive interference conditions. These intensities may be measured directly (I_p and I_X) by setting the polars parallel and crossed with either type of microscope, or by measuring with monochromatic light the intensity at the crossed position and at some small angle, ϕ, a few degrees off extinction, taking advantage of the sine square law of Malus (Cf. Jenkins and White, 1957).

$$F = \frac{I_p}{I_X} = \frac{\frac{I_\phi}{I_X} - 1}{\sin^2 \phi} \qquad \text{.....(6)}$$

In the polarizing microscope, polarizing prisms or filters may be found with extinction factors as high as

10^6, but the addition of lenses and compensators inevitably reduces the extinction factor to 1-2 X 10^4 even in rectified lens systems (Cf. Inoué and Hyde, 1957). Factors chiefly responsible for loss of contrast are strain birefringence and defects in lenses, dust on optical surfaces and internal reflections. Unrectified optical systems may suffer very severe loss of contrast due to depolarization at high numerical apertures. Despite these limitations, there is sufficient contrast in a highly refined optical system ($F = 10^4$) to detect retardations as small as 0.35 Ångstroms (λ/ 15,000 for green light) in minute objects (Inoué and Koester, 1959).

In the interference microscope, the extinction factor is typically 2-3 orders of magnitude lower than in the polarizing microscope. The relatively poor contrast is attributable mostly to the fact that light waves brought to focus at any point in the microscopic field travel different optical paths. For this reason, the extinction factor is highest (and the resolving power poorest) at small apertures and contrast drops sharply as the condenser aperture is increased. At first sight it might seem that signal to noise ratios for measurements would be much lower for interference microscopes than for polarizing microscopes. The increased noise is offset, however, by the correspondingly larger phase retardations usually encountered in objects of biological interest.

The measurement of phase retardations by photometry in the image plane can be considered as a general problem

irrespective of whether they are measured in the interference or the polarizing microscope. The following derivation for image contrast as a function of the retardations of the object and background and of the extinction factor applies to the following situations:

1. Polarized light microscopy with the object axes set at 45° to the plane of incident linearly polarized light, and with the background retardation introduced by compensators of the Ehringhaus, Soleil-Babinet, and de Sénarmont types.

2. Polarizing interference microscopy with the object in a background of uniform (but variable) optical path difference, and a compensator of one of the types mentioned above.

The intensity (I_{OB}) transmitted by any unit area in the image depends on the intensity (I_p) of the light at maximum constructive interference (parallel polars), the sine square of the sum of the phase differences (in radians) of the object (Δ_{OB}) and background (Δ_B) and on the extinction factor (F).

$$I_{OB} = I_p \sin^2 \left[\frac{\Delta_{OB}}{2} + \frac{\Delta_B}{2} \right] + \frac{I_p}{F} \quad \ldots\ldots(7)$$

The corresponding background intensity is

$$I_B = I_p \sin^2 \left(\frac{\Delta_B}{2} \right) + \frac{I_p}{F} \quad \ldots\ldots\ldots\ldots(8)$$

The contrast due to phase retardation differences between the specimen and its background is defined as the differences in their brightness divided by background brightness. This contrast can be shown to depend only on the two phase retardations and on the extinction factor:

$$C = \frac{B_{OB} - B_B}{B_B} = \frac{I_{OB} - I_B}{I_B}$$

$$C = \frac{\sin \frac{\Delta_{OB}}{2} \left[\sin \left(\frac{\Delta_{OB}}{2} + \Delta_B\right)\right]}{\sin^2 \frac{\Delta_B}{2} + \frac{1}{F}} \quad \text{.....(9)}$$

Figures 1 and 2 are plots of the contrast expected for objects exhibiting different phase retardations (Δ_{OB}) at different background retardations (Δ_B) under high and low extinction factors respectively. Figure 1 is representative of the conditions that might be expected in a high extinction polarizing microscope ($F = 10^4$), while figure 2 approximates conditions in an interference system ($F = 50$).

These curves suggest the following comments:

1. On the negative contrast[1] side of these curves (to the right of the origin) the bias retardation (Δ_B) for maximum bright on dark background contrast must be increased if the extinction factor is low. Conversely, in an ideal system, contrast would be maximal (and infinite) with no bias retardation. Contrast curves published by Hale (1958) showed this feature because the extinction factor was not considered.

---

[1]By tradition, microscopists refer to images of a bright object on a darker background as "negative contrast". According to our derivation it appears on the graphs as positive values (above the origin).

2. The maximum positive (dark on light) contrast is found at a bias retardation ($-\Delta_B$) somewhat in excess of Δ_{OB}, that is, just past the extinction point. This is so because the background near the object extinction setting is increasing in brightness more rapidly than the object. This setting is a favorable one for photography (especially cinematography) in polarized light, since it provides a more reliable exposure setting and more total light striking the film plane, as well as maximum contrast.

3. For very small values of Δ_{OB}, the positive and negative contrasts are approximately equal at their respective maxima. As the value of Δ_{OB} increases, the negative contrast becomes much greater.

The compensator is used in different ways: on the one hand, to enhance contrast in order to demonstrate differences in phase retardation, and on the other hand to measure those differences. In the former case, the compensator is set at some small bias angle determined by the object retardation and extinction factor (Figures 1 and 2). In the case of measurements, the compensator is set to meet one of the following conditions:

1. <u>The extinction point</u> is the compensator setting at which the object passes through its darkest shade, i.e. a phase retardation of zero or the null point. This setting is free from all systematic error due to contrast from other optical properties, but the setting itself is inherently imprecise, as the contrast changes very slowly about the extinction point. The precision of the setting

may be improved considerably by the use of a half-shade device permitting two halves of the field of view to be matched in brightness.

2. The match point (except in the case of half-shade eyepieces) is the compensator setting at which the contrast between the object and background is abolished. The match-point can be set far more precisely than the extinction point because the contrast changes very sharply with small changes in optical path. However, this method is potentially misleading; it should be avoided unless it is known that the brightness of the object and background are equal at their respective extinction points. As Bear and Schmitt (1936), showed, specimens outlined in polarized light microscopy are transparent enought that the match-point setting may be considered the most reliable.

3. Photographic densitometric measurements of phase retardations depend on whether contrast is being used to determine extinction or match points or whether contrast is to be used directly as the measure of phase retardations. In the latter case, Allen and Nakajima (1964) have suggested a two-photograph method for polarized light microscopy in which small retardations could be measured from the photographic density difference read from two photographic negatives, one taken on each side of the extinction point, at a compensator angle chosen to equalize photographic contrast as a function of density difference. In dealing with larger path differences it is preferable to set the background retardation (Δ_B) at some value close to $\lambda/4$,

since the contrast on both sides of that point is almost a linear function of the object phase retardation (Δ_{OB}). (e.g. see Hale,1958). In practice it is always necessary to make identical measurements on both sides of the extinction curve, in order that errors due to contrast from absorption, light scattering, etc. will cancel out. The inclusion of an object with a large phase retardation (e.g. 1 λ or more) in the field of the photograph will provide a record of the extinction factor, provided the latitude of the film is sufficient to include the entire sine square intensity function on the linear portion of the sensitometric curve. If the extinction factor is so high that this is not possible under the conditions of film development, then some other calibration procedure must be followed. Various ways of doing this are discussed at length in the books by Hale (1958), and by Krug et. al. (1961), and in papers by Davies (1958), and by Allen and Nakajima (1964).

4. If a photocell or photomultiplier is used to set the compensator, the same types of measurement cited above may be used. The most reliable is still the extinction point setting, even though it is not very sensitive. The sensitivity may be increased by a symmetrical angle technique in which the intensity is read at several settings on both sides of the extinction point, and the latter is estimated by folding a graph of the curve obtained to interpolate the site of the point of minimum brightness. This is equivalent to using a simple phase-modulated light

technique. The methods to be described in the next section employ the same principle of comparing intensities on the sides of the extinction point, but they do so much more rapidly and with far greater precision.

From the foregoing, it follows that the contrast attainable in a given experimental situation is clearly limited by the extinction factor of the available instrument, especially in the interference microscope. Further, most specimens absorb, scatter and depolarize some of the incident light, so that under actual measuring conditions, the extinction factor, and therefore the contrast-generating efficiency of a given system, is reduced even further. The maximum attainable contrast for a given set of instrumental conditions is the prime factor limiting the absolute sensitivity of a measurement with either type of microscope.

If any method of measurement other than the determination of extinction points is to be used, there is the further difficulty of separating the contrast due to retardation from contrast due to other optical properties. This difficulty will be referred to below as the lack of contrast specificity of a particular microscope (e.g. polarizing microscope) for a particular property (in this case, phase retardations due to birefringence).

The time required for a measurement also presents a limitation. A visual measurement of the phase retardation of a specimen detail usually takes about a minute. Exposing two photographs from which a measurement will later

be made densitometrically may take less than a minute, but the additional time required to obtain quantitative data from the film by photographic densitometry may be considerable.

Spatial resolution is a limitation in any type of microscope. This is particularly true of interference microscopes which characteristically have low working apertures.

It has been pointed out especially by Ingelstam (1957) and by Ingelstam and Johanssen (1958), that simultaneous high resolution and high contrast are incompatible goals. Therefore, high sensitivity and high resolution are to the same extent incompatible, since sensitivity depends on contrast. In the polarizing microscope not only is there a loss of contrast at high working numerical apertures, but an anomaly in the diffraction image produces both spurious resolution and spurious contrast unless polarization rectifiers (Inoué and Hyde, 1957) are used. It has been shown, however, that this limitation does not apply in the use of the phase modulated light methods to be discussed (Allen et al. 1963).

B. THE GENERAL APPROACH OF PHASE-MODULATED LIGHT SCANNING METHODS

It is probable that many microscopists faced with the problems of making reproducible compensator settings in interference and polarized light microscopy have discovered independently the advantages of phase-modulation techniques. Experienced microscopists learn that a com-

pensator can be set with the greatest reproducibility either by using a half-shade device or by turning the compensator back and forth to positions of equal brightness and then setting the extinction point "by feeling" the midpoint between these settings (See Inoué and Koester, 1959).

The approach of phase modulation techniques is to make these brightness comparisons not simultaneously, but by repeated comparisons over a period of time. The result is a method similar to mechanical chopping with a rotating sector or a vibrating reed, except that instead of making direct photometric comparisons, the retardations of the object and the phase modulator are first summed and subtracted. The resultant intensity modulation is used either to measure the phase retardation of the object directly as in the spinning compensator method of Allen and Rebhun (1959), or it may be used as a null detector as in the more recent automatic compensation method of Allen, Brault and Moore (1963) in which no moving parts are used.

There are numerous advantages in phase modulation methods. First, the basic detector may be used to detect or measure any of several quite different optical properties, depending on the arrangement of optical components in the system. Figure 3 shows several arrangements to be described in the section to follow which permit the observer to select whether the output of the instrument measures birefringence, refraction, optical rotation, or dichroism.

Second, measurements can be performed with unprecedented sensitivity, precision and speed in very small specimen areas.

Third, the residual errors due to physical properties other than the one selected (Cf. Fig. 3) are very much smaller than when image contrast methods are used.

Finally, with phase modulation methods it is possible to avoid entirely the use of image contrast methods and their pitfalls, and instead to perform null point measurements such as weighted mean phase retardations in selected areas of the image. The specimen may be:

1. placed in the path of a microbeam of phase modulated light for a spot measurement,

2. driven systematically over the microbeam to produce a spatial scanning record,

3. scanned over a selected area for a period of time to detect changes of the specimen with time, or

4. the same measurement (spot, spatial or temporal) may be repeated at a series of wavelengths for spectral scanning, or repeated for various wavelengths at time intervals.

2. THE THEORY AND APPLICATION OF PHASE MODULATION METHODS

A. THE MEASUREMENT OF PHASE RETARDATIONS DUE TO BIREFRINGENCE

The basic optical components of the birefringence detection system (which Allen et. al. 1963 have called the BDS) are: the light source, a polarizer, an electro-optical light modulator (EOLM), strain-free optics, the specimen, an analyzer, and the photomultiplier detector (Fig. 3). For convenience a quarter-wave plate may be added to the system between the specimen and the analyzer.

The microbeam with which a specimen is examined in phase modulated light is formed by an aperture placed close to the field diaphragm of a lamp adjusted for Köhler illumination. This aperture may be imaged directly in the specimen plane by an inverted objective serving as the condenser if the aperture is placed one tube-length or less in front of its shoulder.

The EOLM is a crystal of deuterated KD*P (potassium di-deuterium phosphate) cut perpendicular to its optical axis and mounted so that its optical axis and that of the system are colinear. Modulation of the light intensity passing the analyzer is accomplished by applying a voltage to the polished crystal faces, i. e. parallel to the optical axis. The crystal exhibits an electrically-induced phase retardation, called the Pockels effect, which is proportional to the applied voltage, (Billings 1949_a, 1949_b, Carpenter 1950). If the induced crystalline axes of the EOLM are oriented at 45 degrees from the plane of polarization, the light intensity (I) passing the analyzer follows

the relation for any uniaxial crystal at that orientation.

$$I = I_P \sin^2 \frac{\Delta}{2} \quad \text{.....(11)}$$

where Δ is the phase retardation (in radians; see Equation 4) and I_P is the intensity passing parallel polars.

One function of the EOLM is to introduce positive and negative phase retardations in response to square-wave voltage modulation applied to it. The photomultiplier does not respond to these phase shifts per se, but to changes in light intensity passing the analyzer. As shown in Figure 4, the crystal axes of the EOLM are interchanged during the positive and negative phases of the voltage cycle, so that the corresponding phase changes are of opposite sign. Since the AC voltage modulation is symmetrical about zero, the output of the photomultiplier is rectified square-waves of photocurrent.

When a birefringent specimen is introduced into the light path with its axes parallel to the axes of induced phase retardation in the EOLM (45° from the plane of the polarizer), the modulator and object retardations (Δ m, Δ o) add during one phase of the voltage cycle (equation 12) and subtract during the opposite phase (equation 13):

$$I_1 = I_P \sin^2 \frac{\Delta m + \Delta o}{2} \quad \text{.....(12)}$$

$$I_2 = I_P \sin^2 -\frac{\Delta m + \Delta o}{2} \quad \text{.....(13)}$$

The change in intensity ΔI in the opposite phases of the AC cycle occurs at the fundamental frequency to which the lock-in amplifier is sensitive (3Kc in the case of the

instrument described in Section 3).

That change is given by

$$\Delta I = I_1 - I_2 = I_P \sin^2 \frac{(\Delta_m + \Delta_o)}{2} - \sin^2 \frac{(-\Delta_m + \Delta_o)}{2}$$

$$= I_P \sin \Delta_m \sin \Delta_o \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (14)$$

and could be used directly as a measure of the retardation, due to the specimen but there would be chances for error if variations occurred in source intensity, amplifier gain, or photomultiplier efficiency. However, these difficulties are almost entirely avoided by making the system a null-sensing device. This is done by using the EOLM as an automatic compensator in a servo loop. As Figure 4 shows, the change in intensity (equation 14) produces an asymmetrical photocurrent wave at the fundamental frequency of the lock-in amplifier. The lock-in amplifier responds with a D. C. voltage; this is amplified and returned to the EOLM as negative feedback to restore the net retardation <u>almost</u> to null. The error depends on the extent to which the retardation due to the specimen is not cancelled, and therefore on the loop gain. The instrument is designed to maintain the gain in the servo loop at 100, so that the error will be 1% (the reciprocal of the loop gain). This is accomplished by a second feedback loop designed to maintain the photomultiplier anode voltage at such a value that the photocurrent will remain constant. An error greater than 1% can occur only if the light intensity falls so low that the gain-control loop cannot maintain the servo loop gain at 100.

(In the present instrument, a test-voltage can be injected any time to measure the loop gain directly, so that any necessary corrections may be applied.)

The net effect of the automatic compensator retardation (ΔC) is to return the net retardation to within 1% of null, and therefore restore the intensity modulation signals to near equality (Figure 4).

$$\Delta I = I_o \sin\Delta_m \sin(\Delta_o + \Delta C) \quad \text{.....(15)}$$

At null, $\Delta I = 0$, and therefore $\Delta C = -\Delta o$.

The advantages of measurements made with the EOLM under the null condition with modulated light are: (a) the compensator scale is linear, (b) the measured value is independent of changes in source brightness, modulation voltage, amplifier gain, and photomultiplier efficiency, and (c) the measurements are also insensitive to moderate amounts of stray light, either from the instrument or from light-scattering by the specimen.

The BDS is first used to identify the crystalline axes of the specimen, or some selected detail thereof, by finding the angular position (called the null angle) at which the reading for the specimen equals that of the background. The retardation due to the specimen is then measured by turning the specimen stage 45^o to the position where the maximum retardation may be read from the meter, recorder, or oscilloscope. The sign of the birefringence is given directly by the sign of the voltage if the long axis of the object is oriented at $+45^o$ from the plane of the polarizer. (The polarity of the instrument should be

verified once with a compensator, the axes of which have been checked by interference colors and marked.)

Certain precautions should be taken in the measurements themselves and in the interpretation of them.

a. It is important to remove as many sources of stray phase retardations as possible, and to orient lenses with strain-birefringence parallel to the crystalline axes of the EOLM. The "mirror-compensator" should be adjusted to compensate for phase shifts due to mirrors and lenses. If this is not done, the BDS may respond to rotation of the analyzer and to optical rotation in the specimen.

b. It is possible to err in determining the orientation of the cyrstalline axes of strongly rotatory crystals (a situation probably never encountered in biological material). The usual procedure is to rotate the specimen until the null angle is reached; this is defined as the orientation at which the specimen and background read the same value - zero if the instrument is properly adjusted and the container is free from strain birefringence. The maximum retardation reading of the BDS, which in non-rotatory specimens is found at 45^{o} from the null angle, will not be found at exactly 45^{o} in rotatory specimens. However, the value at the 45^{o} setting will not be in error by more than 1% if the crystal rotates the plane of polarization by less than 4^{o}. In the latter case, a double lock-in system for detecting optical rotation in the presence of birefringence can be used (Section II. C). In this case,

however, a valid estimate of the retardation due to the specimen can be otained only by turning the analyzer so as to null the signal in the rotation channel, since the phase retardation channel is insensitive to the compensating rotation which is introduced by the rotation servo before the second EOLM (for the phase retardation channel).

c. It can be shown that linear dichroism has no effect either on the determination of crystalline axes or on the measured retardation at 45^{o} off the null angle, provided the phase retardation and dichroic axes coincide.

d. Circular dichroism (the differential absorption of right and left circularly polarized light) would be registered as a phase retardation independently of specimen orientation. It appears doubtful that this effect would be detected in specimens of microscopical dimensions, with the possible exception of crystals.

e. It must be borne in mind during the interpretation of microbeam scanning data that there may be unresolved microdomains contributing in different ways to the final measurement. If these microdomains are weakly birefringent and are oriented parallel to one another but differ in retardation (e.g. a myofibril), the final measurement will be a mean retardation weighted by the areas. If the microdomains are highly birefringent and randomly oriented within the area of the microbeam, it might be erroneously concluded that the specimen was isotropic. It is therefore desirable to scan with the smallest spots over areas of particular interest. If the microdomains

are preferentially oriented, as seems probable in many instances, there is no way in which to analyze them in an exact manner in the absence of additional information on the statistics either of the orientations or retardation of the constituent microdomains. If some microdomains are overlaid by absorbing bodies, their retardations will not receive proper weighing by area as is usually the case.

This list of possible measurement and interpretation errors is not different from that which could be made for any method of polarized light microscopy. The reason for including it here is that the gains in sensitivity and precision obtained with methods based on phase modulated light may lure investigators into placing excessive confidence in the intrinsic accuracy of measured numbers. With conventional polarizing microscopes, measurement errors are sufficiently great that second-order-effect errors need not be considered.

B. THE MEASUREMENT OF OPTICAL ROTATION

The components of the 'birefringence detection system' BDS) will respond to rotation of the analyzer, and therefore to optical rotation in specimens, if the EOLM is followed immediately by a quarter-wave plate with its crystalline axes parallel to the polarizer transmission plane. In this arrangement, which will be referred to as the 'optical rotation detection system' (ORDS), the EOLM - quarter-wave plate combination acts to swing the

plane of polarization around the zero setting of the polarizer when positive and negative phase shifts are induced by the AC voltage modulation to the EOLM. (See also Takasaki, 1961a, 1961b, 1962). The light intensity passing the analyzer varies with the angle (θ) of the analyzer from the extinction position by a sine-square relationship similar to that given in equation 1 for phase retardations:

$$I = I_o \sin^2 \theta \quad \ldots\ldots\ldots\ldots\ldots\ldots (16)$$

In any polarimeter based on vibration of the plane of polarization (e.g. Takasaki 1961, Cary et al. 1964, and others), the rotations of the modulator and sample simply add and subtract exactly as in the case of phase retardations, and the modulation patterns and compensator function are analogous to those shown in Figure 4. The lock-in amplifier responds in the same way as for phase retardation measurements, except that the phase shifts induced in the EOLM are analyzed by the quarter-wave plate analyzer combination into optical rotation. Thus the EOLM in this case acts as a compensator for rotation. This is optically equivalent to rotating the analyzer, and has the advantage that electrical measurements are the more accurate and rapid.

With this simple arrangement it is possible to make measurements of optical rotation on extremely small samples of fluids under the microscope, provided that the rotation due to the container (the use of a chamber made of two flat, plane-parallel strain-free cover glasses is recommended) is subtracted from that due to the container together with

the sample. Since the normal path length through microscopic objects is of the order of 100 μ rather than the 10 cm path in a polarimeter cuvette, a polarimetric measurement of a microscopic sample must be 1000 times more sensitive. The smallest rotations that can be detected with the ORDS are of the order of 10^{-4} degree. It would thus appear possible to make at least some crude measurements of optical rotation in very small samples of solutions with this method.

C. OPTICAL ROTATION IN THE PRESENCE OF BIREFRINGENCE

To the present time, the measurement of optical rotation has not been possible in birefringent specimens for two reasons. First, birefringence is a much stronger source of contrast than optical rotation. Second, the presence of phase retardations due to birefringence introduces a spurious rotation which cannot easily be differentiated from the rotation intrinsic to the molecular asymmetry of the specimen.

The reason why the presence of birefringence complicates rotation measurements can be seen by considering the plane polarized light incident on a birefringent object that produces a phase retardation Δ and has its fast crystalline axis at an angle $\emptyset$ from the plane of polarization. The emerging light is not only elliptically polarized, but the direction of the major axis of the ellipse has been rotated. If the retardation is small, the rotation (B) of the major axis of the ellipse is

given by:

$$B = \frac{\Delta}{2} \sin 4\phi \quad \ldots\ldots\ldots\ldots\ldots\ldots (17)$$

At any given orientation, such a phase retarding birefringent object is optically equivalent to, and therefore indistinguishable from, a retarding object at 45^{o} followed or preceded a pure rotator, such as an optically active solution. The rotation which accompanies the birefringent object is spurious and can be eliminated by making use of its known angular dependence. The ellipticity introduced by such a plate is of the form

$$E = \Delta \sin 2\theta \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (18)$$

Therefore, by rotating the object to such an angle that the ellipticity vanishes, the spurious rotation is also removed, and the true rotation of the specimen can be measured.

Measurements of optical rotation in birefringent specimens must be carried out using two lock-in amplifiers, each coupled to its own modulator. The first modulator (EOLM No.1) is followed by a quarter-wave plate and serves to swing the plane of polarization over an angle of $\pm$ 20 degrees from the extinction point at the rate of 30 c.p.s.; this modulator serves also as a rotation compensator for the ORDS. The second modulator (EOLM No. 2) is driven at 3 k.c.s. as a normal phase-modulator and automatic compensator of the BDS. Except for reciprocal contributions of noise, the two channels (the ORDS and BDS) operate independently to detect rotation and phase-retardation

respectively.

In the simplest case of a birefringent container with an optically active solution, the proper method to measure rotation would be to rotate the container on the stage, noting that the rotation channel records four maxima at angles midway between the positions at which the crystalline axes are either parallel or diagonal to the polarizer. If the stage is set in the position at which the readings for phase retardation of the object and the background are the same, the rotation chennel will read the correct optical rotation, provided strain birefringence from the cover-glasses has not entered into the measurement. Since these strains tend not to be highly localized, a check against this possibility is easily made by repeating the measurement on a few neighboring background areas.

It is also possible that the rotator may be aligned. In this case the helices or partial helices which may be considered to be causing rotation may be oriented parallel to the light beam, perpendicular to it, or at some intermediate angle. It should be borne in mind that the rotation will be strongest if the light is parallel to the helical axis.

It is possible to measure rotation not only at the null angle but also at 45^{o} from null. This setting may be of some interest in biology, since it renders possible the simultaneous measurement of changes of birefringence and rotation.

Optical rotatory dispersion (ORD) measurements are also

possible in principle, but it seems doubtful at present whether sufficiently accurate measurements could be obtained for dispersion curves on thin microscopic objects. If such measurements are to be attempted, they should be made with the brightest available light source at several wavelengths for which interference filters are available, or better still with a monochromator (see Djerassi, 1960, for a discussion of ORD methods). The EOLM phase retardation, measured in Ångstroms per volt of applied potential difference is invariant with wavelength, but the angular phase shifts and their rotatory equivalents do vary. The true rotation at some arbitrary wavelength λ will equal the observed rotation multiplied by $546/\lambda$, since the calibration of the instrument was carried out for the mecury green line.

If the faster (3 k.c.) birefringence channel is used for estimating rotation the following formula to convert Ångstroms to degrees is as follows:

$$\text{Rotation angle in degrees} = \frac{\Gamma \text{ in angstroms}}{\lambda \text{ in angstroms}} \times 180^{\circ}$$

It should be apparent from the foregoing that any method which responds to analyzer rotation should respond to the rotation of a dichroic specimen on the object stage with the analyzer removed (Cf. Fig. 3). In point of fact, however, the arrangement shown in Figure 3 is more convenient as a detector of dichroism than as a measurement method. It is possible to detect a difference in absorption of one part in a thousand or less. Dichroic ratios can be more conveniently measured by a double-beam phase modulation method to be described in a subsequent paper.

D. THE MEASUREMENT OF PHASE RETARDATIONS DUE TO REFRACTION WITH A POLARIZING INTERFERENCE MICROSCOPE

In Section I it was pointed out that phase retardations in polarizing and polarizing interference microscopes are physically similar and therefore may be measured with basically the same image contrast and phase modulation methods.

In interference microscopy the range of phase retardations encountered in biological material (e.g. tissue culture cells or protozoans) is at least twice (and often several times) as large as the range of phase retardations due to birefringence found in polarization microscopic examination of the same material.

Retardations greater than one wavelength are best measured in white light using a recognizable interference color for reference in order to avoid errors due to choosing the wrong fringe for setting the extinction point (CF. Bennett 1950, Piller 1962, Gahm 1962, 1963, and Krug et. al. 1961). The most useful compensators for retardations greater than one wavelength are the Ehringhaus and Soleil-Babinet plates. Retardations of less than one wavelength are most conveniently measured with the de Sénarmont method, although the analyzer cannot be set so accurately as with a Köhler rotating mica plate.

The BDS described in detail in Section II. 1 (See also Allen et al. 1963) can be applied to interference microscopy without any major modifications, beyond replacing the strain-free objectives with interference optics. One

necessary modification is that the aperture for the measuring microbeam must be imaged in the plane of whatever field stop is normally imaged in the specimen plane. Thus in the Zeiss interference system, a lens must be inserted in position 16 in Figure 5 in order to image the aperture in the plane of the front diaphragm (20 in Figure 5).

Dyson (1963) has recently reported the use of phase modulated light and a phase-sensitive dectector as a null-detecting aid in setting the analyzer in spot measurements with a polarizing interference microscope. From our experience it seems likely that the precision of the null setting would be considerably greater than that of the analyzer reading. For this reason, the use of the EOLM as an automatic compensator is attractive for its precision as well as its speed and convenience.

The major limitation of the BDS as applied to interference microscopy is its range, normally $\lambda/4$. This range may be doubled by placing two EOLMs in series separated by a half-wave plate with its crystalline axes parallel to the original plane of polarization. This arrangement not only doubles the range (to $\lambda/2$), but eliminates most of the noise due to fluctuations in the spatial position of the light source[1]. The deflection

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[1] If the EOLM is made from a birefringent crystal (e.g., KOP, KDP, ADP, etc.) noise will result from any motion of the light source, EOLM, or photomultiplier. If two EOLMS are used with a $\lambda/2$ plate between them, noise signals due to motion of the light source are of opposite sign in the two modulators and therefore are reduced.

sensitivity is, of course, halved, and the BDS must then be recalibrated, (Section 3C).

The BDS is most useful for scanning measurements on small objects with phase retardations considerable smaller than $\lambda/2$. The maximum error in the phase retardations measurement itself is the servo error (1%) until the scale is expanded to the point that photon noise or noise due to light source wandering (especially with mercury arc sources) becomes evident. The noise depends, as in birefringence measurements, on the microbeam diameter and time constant, but is lower by a factor of 10-100 than the uncertainty in compensator setting by conventional contrast methods. With a spot 8 μ in diameter in a 40X lens system (or 3.2μ in a 100X system) and a time constant of one second, the noise level with a condenser numerical aperture of 0.3 was 0.1 A or e.g. $\lambda/50,000$.

The BDS can also be used to improve the precision of measurements of retardations in excess of a half or even several full wavelengths by using it to set the extinction point with a suitable manually operated compensator, once the fringe of the proper order has been identified with heterochromatic light. While the precision with which the extinction position is set may be $\lambda/50,000$ or better, the compensator probably cannot be read to better than $\lambda/1800$.

The usefulness of the BDS for interference microscopy will be greatly increased when modulators with a greater range become available.

The uses of the interference microscope are already many, and it may be hoped that the greatly improved sensitivity and precision offered by phase modulation techniques will provide many opportunities in cytology and cytochemistry in particular, where the interference microscope has already permitted a number of significant advances both in technique and knowledge gained. (Cf. Barer 1952, 1953, 1956,1957; Barer and Dick 1955; Barer and Joseph, 1954, 1955; David 1964; Davies 1958; Davies and Wilkins 1952; Dyson 1957; Françon 1961; Gahm 1962, 1963; Hale 1958, 1960; Horn 1958; Huxley 1952; Ingelstam 1957; Ingelstam and Johansson 1958; Krug et al. 1961; Pehland and Hager 1959; Piller 1962; Smith 1955.)

It is perhaps worth reviewing and commenting on the types of measurements and applications now possible with the interference microscope to see what possible advantage may be gained through the greatly enhanced sensitivity of phase modulation methods.

1. The refractive index of a specimen (n_o) can be measured if its thickness (t) and the refractive index of the surrounding medium (n_m) are known:

$$n_o = \frac{\Gamma}{t} + n_m \quad \text{.....(19)}$$

2. The refractive index of the specimen can be measured independently of its thickness if measurements are repeated with two media of different refractive index (n_{m1} and n_{m2}).

$$n_o = 2\,\frac{n_{m1} - n_{m2}}{\Gamma_1 - \Gamma_2} + n_{m2} \quad \text{.....(20)}$$

3. Thickness may be determined if the refractive indices

of the object and medium are known by solving for (t) in equation (19).

4. If the refractive index of a cell or one if its parts is known, the dry organic matter concentration (W/V) (C) can be estimated from the fact that the specific refractive increment (α) of the macromolecules and small organic molecules most prevalent in living material is approximately 0.00185 (Davies 1960).

$$C = \frac{n_o - n_w}{\alpha \times 100} = \frac{n_o - n_{H_2O}}{0.185} \quad \text{.....(21)}$$

5. The dry organic mass in grams of a single cell or even of a single inclusion in water may be estimated from a weighted mean retardation measurement ($\bar{\Gamma}$) and a planimetric determination of the area (A) over which this mean was measured.

$$M = \frac{\bar{\Gamma} A}{\times 100} = \frac{\bar{\Gamma} A}{0.185} \quad \text{.....(22)}$$

According to David (1964), contrast methods of interference microscopy permit the estimation of dry mass in objects containing as little as 10^{-15}g of solids. Phase modulation methods should improve this figure by at least one order of magnitude.

If the value of $\bar{\Gamma}$ can be obtained for two media of different refractive index, or if the dispersion of the specimen and medium differ or can be differentially affected by temperature (Cf. Pehland and Hager 1959), it is then possible in principle to estimate refractive indices, thickness, dry mass volume, and wet weight, all without having to immerse objects successively in two media (Hale 1958).

Phase-modulation methods now offer for the first time ample sensitivity and precision for testing the limits of interference microscopy as a quantitative technique. The fact that the output value is a weighted mean phase retardation should be of considerable advantage in dry mass determinations, for this is exactly what the theory of the method calls for.

It should also be pointed out that measurements with any interference microscopes are subject to certain systematic errors. These do not appear particularly significant in conventional methods which are limited by stray light noise at the level of $\lambda/100$ to $\lambda/500$. Small systematic errors become at once more significant if the sensitivity of measurements is increased by 10-100 times.

The most serious systematic error is really one of interpretation, and it results from the fact that light waves entering a specimen at different angles to the optical axis of the microscope travel different optical paths in the specimen, depending on the shape of the specimen. The error is greatest, for example, with a flat specimen and least with a spherical one. The flat specimen will register a higher phase retardation as the iris diaphragm of the condenser is opened. The error may amount to as much as 10-15 %.

The most serious potential source of random error is probably contamination of the reference beam area. This is important to consider in measurements with

phase-modulated light, since particles too small to be seen can introduce phase retardations and therefore errors. It is best, therefore, to scan the object more than once, using different empty areas for the reference beam.

It is particularly important to bear in mind that both the sample and reference beams are linearly polarized. Advantage should be taken of this fact to determine the refractive indices for the crystalline axes of birefringent specimens (Cf. Bruce and Thornton 1957, Gahm 1962). The difference in refractive indices is the birefringence (equation 2).

The use of phase modulation appears to represent a significant advance over the integration methods for dry mass, such as that of Davies and Deeley (1956), which relies on photometry along the relatively linear portion of the sine square contrast curve. The reliance on null point measurements of weighted mean phase retardations instead of straight photometry in the image plane would be expected to diminish errors due to lack of contrast specificity. The superior noise rejection of the lock-in amplifier over that of a simple demodulator is probably responsible in large part for the improvement in sensitivity of the present technique, which is estimated as one order of magnitude or more.

3. THE DESIGN OF AN INSTRUMENT FOR QUANTITATIVE MICROSCOPY - THE PAR SYSTEM

A. THE OPTICAL SYSTEM

An important weakness of most scanning microscopes is the loss of the whole image and with it the knowledge of what part of the specimen is being scanned by the microbeam. The PAR SYSTEM * was designed to avoid this

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* Design and construction by the Princeton Applied Research Corporation. We are grateful to Dr. Horst Piller of the Carl Zeiss firm for arranging the special manufacture of certain essential parts of the system (nos. 6, 15, 28 in Figure 5).

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weakness by the provision of a dual illumination system that allows the specimen to be observed or photographed as scanning data are gathered electrically. The PAR SYSTEM is described below in detail as an example of an arrangement designed to take advantage of phase-modulation techniques.

The way in which this dual illumination was achieved is shown in Figures 5 and 6, a diagram of the modified Zeiss Photomicroscope. Light from a bright source (1) such as a zirconium oxide, mercury, or xenon arc is passed through a field lens (2), infra-red absorbing filter (3), an interference filter (4), and illuminates either a circular or slit-shaped aperture (5). This aperture is imaged by an achromatic lens No. 1 (16) in the plane of the distal field-stop (17) and it is re-imaged in the plane of the specimen by the microscope

condenser (22), which may be an inverted strain-free objective. An alternative lens (No. 2) for position (16) images the aperture in the plane of the proximal field-stop (20). The microbeam transmitted by the aperture passes through the modulator unit which consists of a polarizer (7), either one or two electro-optic light modulators (EOLM's) (8,10), and a quarter- or half-wave plate (9) depending on the application. Three first-surface, quarter-wave flat aluminized mirrors (6,15,19) all introduce small phase shifts which can be compensated by the mirror-compensator (11). Mirror (15) may be a dichroic beam-splitting element allowing short wavelengths from the microbeam to be reflected into the optical axis of the microscope and transmitting long-wavelength unmodulated light from the auxiliary tungsten lamp, (12) into the same path. Otherwise this element (15) may be a 70-30 reflecting-transmitting beam splitter. Lenses (16 and 18) and one cover-plate (21) must be strain-free and of good optical quality.

When the consenser (22) is focused on the distal field-stop (17) the background of the field of view is illuminated by an out-of-focus red image of the field stop (14) of the auxiliary tungsten lamp (12). In order to attenuate the short-wavelength unmodulated light, a red filter may be placed after the auxiliary tungsten lamp field lens (13).

Modulated light from the microbeam and unmodulated light with which the rest of the specimen may be observed

are both transmitted through the microscope objective (24), the telan lens (25), and analyzer (26), and optovar magnification changer (27). The beam splitter of the photomicroscope head has two positions which may be used with the PAR SYSTEM. Position 3, (black) provides a neutral beam-splitter which sends 50% of the light to the photomultiplier and 50% to the eye-piece facing the observer. Position 2 (red) provides a dichroic beam splitter (28) which directs short-wavelength light (from the microbeam) to the photomultiplier (31). Positions 1 and 4 of the beam-splitter are for observation and cinematography, respectively, and do not allow any portion of the light to reach the photomultiplier tube. At position (30) there is a neutral 50% reflecting plate which allows the observer to see either an image of the plane of the film in the 35 mm camera or the photomultiplier lens, depending on which is in place. This beam-splitter is removable to permit the signal level at the photomultiplier to be doubled. The 35 mm camera and the photomultiplier housings (31) can be rapidly interchanged (see Figure 6).

B. THE ELECTRONIC DETECTION SYSTEM

The two-channel electronic system used in the PAR SYSTEM consists of two fixed-frequency "lock-in amplifiers" (Figure 7 and 8). Each lock-in amplifier operates virtually independently of the other in its own servo loop containing an automatic compensator for each of the optical properties measured. The lock-in amplifier is a phase and frequency sensitive detector capable of amplifying selectively signal of a chosen frequency while rejecting other frequencies (Cf. Dick 1947, Moore 1962).

Figure 7 shows a block diagram of the electronic system with its two channels. The heart of each lock-in amplifier is a phase-sensitive detector which in this case is a synchronous demodulator in combination with a DC band-pass amplifier. The synchronous demodulator is an electronic double-pole, double-throw switch operated at channel frequency (from an internal oscillator signal). A signal at the frequency of the demodulator will be full-wave rectified with the output polarity determined by the phase of the signal. That is, if the signal and the oscillator reference are in phase, the output voltage will be positive. If 180 degrees out of phase, the output will be negative.

The oscillator is of the stable twin-T type with two outputs. The first provides the phase shifter with the

reference channel frequency for the phase-sensitive detector. The second is connected to the AC EOLM-driver.

The phase shifter is required because the maximum output of the phase sensitive detector is dependent on the signal and reference being either exactly in, or 180 degrees out of phase. An adjustable RC phase-shifter variable from 0-90 degrees is provided to compensate for any phase shift within the electronic system and therefore to optimize the demodulator output.

The EOLM-driver is a hybrid transistor-tube (thermionic valve) circuit which amplifies the square-wave output of the oscillator and applies this voltage to the EOLM when the "mode selector" switch is turned to either the "open loop" or "servo" position. The peak-to-peak AC voltage applied to each EOLM is +950 volts, which induces a retardation of about ±750 Ångstroms.

The photomultiplier is an RCA type 1P21 tube with a spectral response between 3500 and 5500 Angstroms (maximum: 4000Å). A conventional operational DC current amplifier has been inserted between the high-impedance output of the photomultiplier tube and the lock-in amplifier. This amplifier has an open-loop gain of 2000 and a frequency response greater than 100Kc, and an output of from -7 to +10 volts. The open loop gain of this amplifier can be varied 10% by the "fine gain control". The "coarse gain control" has two final positions (far

clockwise) which increase the amplifier gain by factors of 3 and 10 respectively. All other positions of the "coarse gain control" vary only the anode voltage (Figure 8).

The "automatic" position of the"coarse control" is designed to minimize servo error by keeping the signal at a constantly high level. At any other position of the "coarse gain control" light losses in the measuring beam would diminish the signal amplitude and the loop gain and therefore increase the servo error. At the "auto" position, the photocurrent is kept constant over a considerable range of light intensities by an auxiliary amplifier which senses the output of the photomultiplier amplifier and varies the power supply voltage to compensate for photocurrent variations.

The DC amplifier is a hybrid transistor-tube circuit which amplifies the DC output of the phase-sensitive detector. When the "mode selector" is turned to "servo", this DC voltage is returned to the EOLM forcing it to serve as a linear automatic compensator. The voltage which appears at the meter, recorder, or oscilloscope is that which is required to bring the optical property under investigation to null. The "response time (sec)" control on the front panel controls the response time of the voltmeter only, and in no way affects the servo speed.

The "meter zero" adjustment applies a $\pm$ 20 percent of range bias voltage to the voltmeter and recorder.

It is not in the servo loop and cannot affect its performance. The meter zero is used to bring the meter indicator to the center in order to expand the scale for small change measurements.

The "system zero" is a bias within the servo loop which may be applied in order to null out the effects of residual retardation. It is properly adjusted when the meter does not change its reading when the mode selector is switched back and forth from "servo" to "open loop" with the gain control adjusted to read ca. 70 percent of full scale.

The "dark current zero" adjustment is provided in order to null the dark photocurrent from the photomultiplier to provide conditions which are optimal for servo performance.

The entire system is sufficiently compact to be mounted on a secretary's desk in which the typewriter drawer is used to house a pen recorder. The operator thus can reach all optical and electronic controls while seated at the microscope. The entire arrangement is shown in Figure 9.

C. CALIBRATION AND ERROR DETERMINATION

1. Phase retardation measurements

The proportionality between the final voltage output of the servo and specimen phase retardation is determined by a simple calibration procedure in which a quarter-wave plate is rotated a few degrees on the stage of the microscope. It can be shown that there is an automatic compensator setting for which there is no change in transmitted intensity when the modulator axes are interchanged as the quarter-wave plate is rotated by an angle θ (in radians) from its extinction point. The phase retardation (Δ_C) of the automatic compensator (the EOLM) is given by:

$$\tan \Delta_C = \frac{\sin 2\theta}{\cos^2 2\theta} \qquad \text{.....(23)}$$

Over four degrees of arc the following approximation is accurate to one percent:

$$\Delta_C = 2\theta \qquad \text{.....(24)}$$

Since a 100Å retardation for light of 5461 Angstroms wavelength corresponds to 0.115 radian, a rotation of the quarter-wave plate of ± 0.0575 radian or ± 3.29 degrees about extinction should drive the meter to the plus and minus full-scale extremes in the 100 Å full-scale range.

The instrument once calibrated either with no objective and condenser in place or with strain-free optics will be already properly adjusted to measure phase retar-

dations due to refraction when interference optics are inserted.

2. Optical Rotation

The optical rotation channel is calibrated by adjusting the meter response potentiometer of the rotation channel until the meter responds full-scale at the proper range to a 10 degree-rotation of the analyzer.

D. DATA REGISTRATION

Three meters are provided on the front panel (Fig. 8) of the instrument for reading the output of both servos and the photomultiplier amplifier. The recorder (Fig. 9) is arranged to make permanent records of any two of these meter outputs simultaneously. The meter and recorder responses are slow, however, so that it is necessary to use an oscilloscope or other rapid recording device for rapid events.

Small changes in phase retardation, rotation, light transmission, etc. can be measured by bringing the desired inspection range into the center of the meter so that a small portion of the range can be greatly expanded. At some point in the scale expansion process noise will appear in the record. If the light source is stable and the instrument properly aligned (especially the leveling of the EOLMs) then the noise will be due almost entirely to photon noise, i.e. statistical fluctuations in the

stream of photons striking the photocathode. Since the magnitude of this noise varies with the reciprocal of the square root of the measurement time, the noise can be supressed by RC filtering. The "response time (sec)" adjustment filters noise from the records, but introduces a time constant (τ). The time constant (τ) is defined as the time required for the voltage output to reach 63 percent of its final value after the system had received a step change. After double this interval (2τ) the voltage has reached 87 percent of its final value. Periodic events will be attenuated by RC filtering. For example, a frequency of $\frac{1}{12.9\tau}$ will be attenuated by 10 percent. A frequency of $\frac{1}{2\pi\tau}$ will be attenuated by 29 percent.

The time constant imposed by whatever filtering is employed must be considered carefully not only in recording periodic phenomena, but in scanning across a specimen with considerable detail. The optical information in this detail may be filtered out if too large a time constant is used.

When the electrical data must be correlated with information about movement or changes in shape of the specimen, it may be desirable to record one or both of the servo outputs on cine film along with a cinematographic record of the unmodulated light image. This is done by

imaging the oscilloscope or recorder surface on the left-hand margin of the ciné film by means of a beam splitter placed between the vertical eyepiece and ciné camera lens. An area corresponding to that in which the electrical record will be stored is first blacked out with dark paper at the plane of the primary image (i.e. where the eyepiece micrometer is placed) in the ocular.

E. LIMITATIONS ON SPEED, SENSITIVITY AND SPATIAL RESOLUTION

1. Speed

The fastest response time of the phase retardation channel of the PAR SYSTEM is 1 millisecond. The possibility of observing small changes in a given optical property occurring in one millisecond or less is diminished by the amount of noise and oscillator harmonics in the servo loop. The practical limit, therefore, would appear to be the amount of noise that can be tolerated while measuring the event of interest.

2. Sensitivity

The deflection sensitivity of the instrument is fixed in the case both of retardation and optical rotation measurements by the electro-optic coefficient of the EOLM.

The absolute sensitivity, i.e. the smallest change in an optical property that can be measured, is determined by the amount of noise in the output record. By plotting the noise as a function of the reciprocal of the micro-

beam spot diameter, it is possible to determine whether the cause of the noise is fluctuations in photon emission (photon noise) or instrumental noise.

As with any instrument of this type, the signal-to-noise ratio is proportional to the square root of the photon flux and is a function of the time constant. Thus minor gains in signal-to-noise ratios may be achieved by substituting more efficient photomultiplier tubes and polars, by using brighter light-sources, and by integrating measurements over longer response times by RC filtering. The photon flux will be determined by the brightness of the light source, the transmission of the specimen, any filters that are used in the path of the measuring microbeam, and the area of the aperture which bounds the microbeam. The numerical aperture of the condenser will determine the divergence angle of the microbeam at the specimen plane and therefore its resolving power.

3. Spatial resolution

If the microbeam is used to scan a specimen with detail, the spatial resolution of recorded measurements will depend on the numerical apertures of the condenser and objective lenses. Thus the limit of spatial resolution obtainable by scanning methods is one Airy unit, or about 0.2-0.3 micron for visible light in the blue and green parts of the specimen. However, it must be borne in mind

that at the limit of resolution (i.e. with the apertures imaged to one Airy unit in diameter), the "leakage" of light from the spot into the diffraction rings amounts to some 16% of the energy in the beam. Phase retardation measurements with a precision of 1 percent obviously do not carry much meaning under these circumstances. It is preferable to choose a minimum spot size at which the percent of the energy loss in diffraction equals the acceptable margin of error.

In the case of polarized light microscopy, it has been shown by Allen, Brault, and Moore, (1) that the anomalous diffraction image in unrectified polarizing microscope optics affects neither the measuring accuracy nor the spatial resolution of the BDS. Therefore rectifiers are not required.

There may be occasions when the symmetry or the periodicity of a biological object may permit scanning with a slit instead of a circular aperture in order to improve the signal-to-noise ratio without loss of spatial resolution. For example, scanning a cylindrical object with a slit 1 Airy unit wide and 10 units long would increase the signal-to-noise ratio more than three-fold with no decrease in resolution across the diameter of the cylinder.

F. CONCLUDING REMARKS

We have presented an account of the theory of phase retardation measurements in polarization and interference microscopy by image contrast and phase-modulation methods. Image contrast methods offer the great advantage of providing the observer with the enormous amount of information in an image and the opportunity to scan this quanitatively with the eye or capture the spatial pattern of phase retardation differences on film. The problems of measuring these phase retardations from images by means of their contrast is at best, however, a difficult task even for a microscopist experienced in the difficulties of photometry.

Phase modulation methods, on the other hand, while not very useful for the preliminary inspection of a specimen, are nearly ideal for making spot measurements or for spatial, temporal, or spectral scans. In modern cellular and molecular biology there are increasing needs for more sophisticated optical techniques to unravel the mysteries of biological organization. Phase modulation methods offer many possibilities for studying light-matter interactions, only a few of which have been discussed here. We have not included in this discussion any treatment of phase modulation methods for absorption, dichroism, fluorescence, or light scattering. We have also postponed

a discussion of the arrangements for the electromechanical scanning of the specimen. These subjects will be considered in subsequent papers.

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Figure Captions

Figure 1. Contrast curves for objects with different phase retardations (Δ_{OB}) at different background (or bias) retardations (Δ_B) for a high-extinction polarizing microscope ($F = 10$). Curves were computed from equation (9).

Figure 2. Contrast curves for objects with different phase retardations Δ_{OB} [or optical path differences, see equations (4) and (5)] at different background (or bias) retardations Δ_B for either an inefficient polarizing system, such as a crude polarizing microscope or a polarizing interference microscope ($F = 50$). Curves were computed from equation (9).

Figure 3. A diagrammatic summary of different optical arrangements that may be used with phase modulated light in polarization and interference microscopy as described in the text.

Figure 4. A diagram to illustrate the basic system for the detection and measurement of phase retardation and optical rotation (see text).

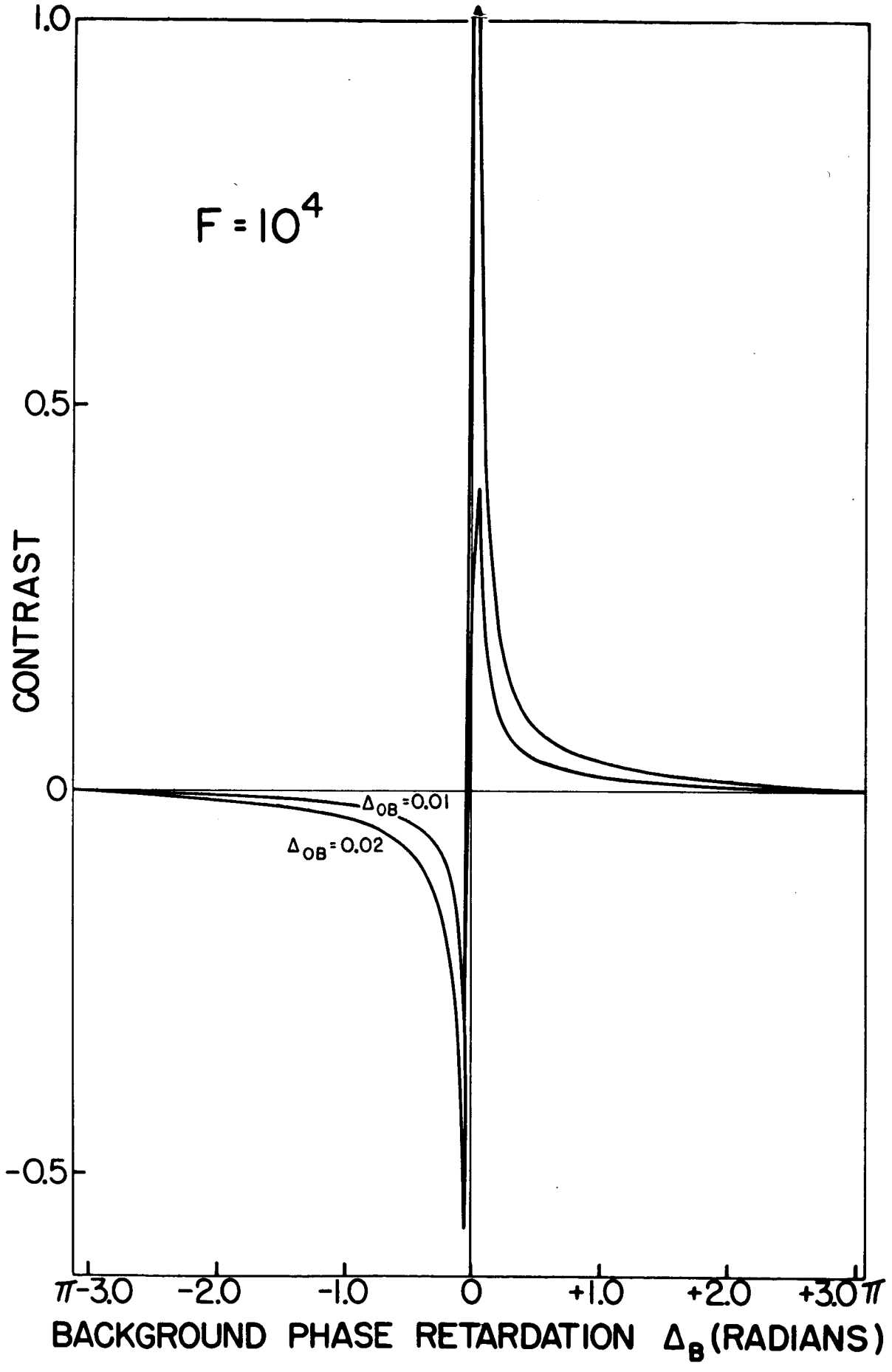
Figure 5. A diagram of the modified Zeiss Photomicroscope (see text).

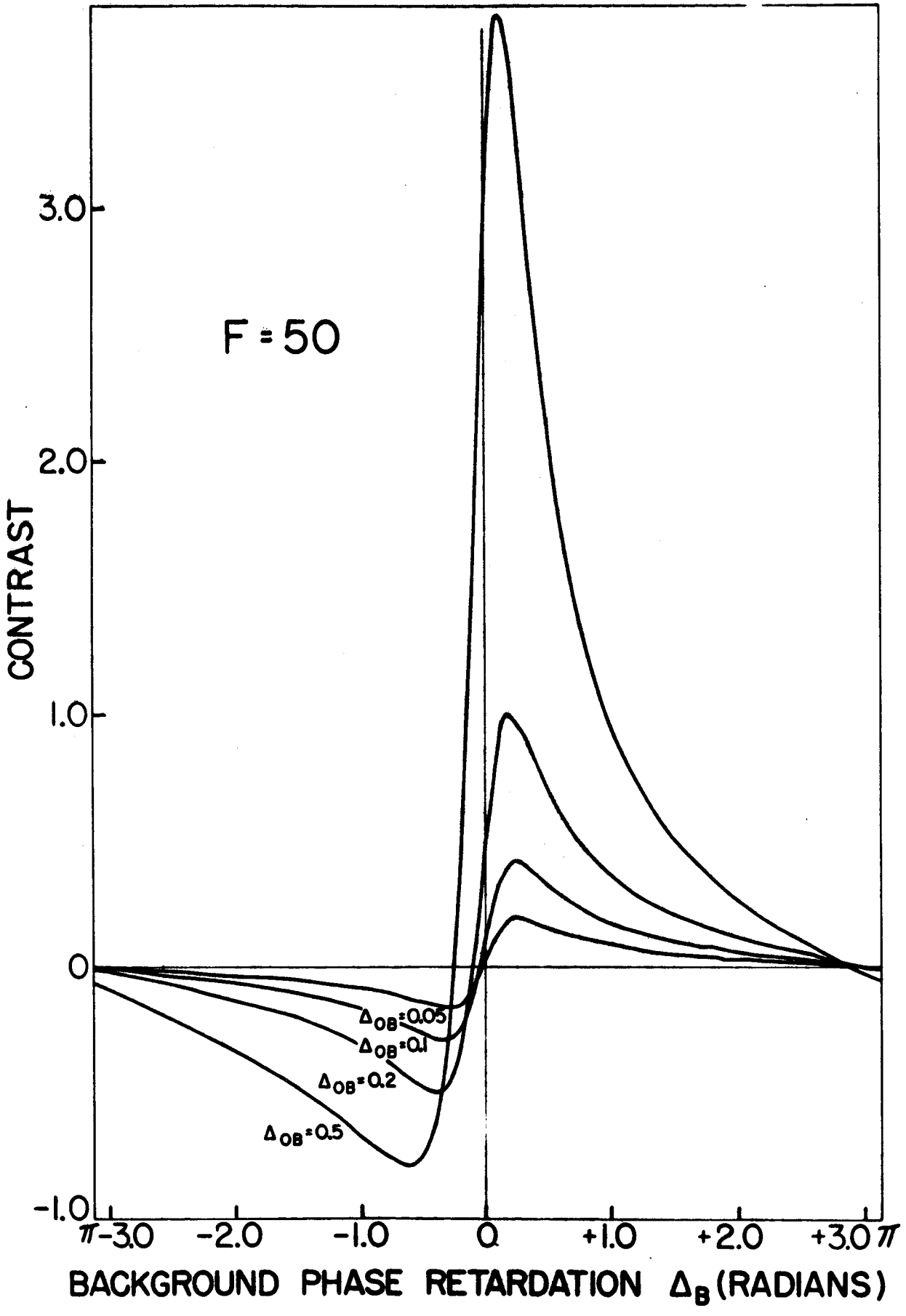
Figure 6. A side view of the modified Zeiss Photomicroscope to show the rotating drums for interference filters (1) and microbeam apertures (2), the modulator assembly (3), the photomultiplier housing (4), and the slide for removal of the 50% beam splitter before the photomultiplier (5).

Figure 7. A block diagram of the electronic system. See text for details.

Figure 8. A photograph of the front panel of the control box of the instrument manufactured by Princeton Applied Research Corporation.

Figure 9. A photograph of the PAR system as used for polarization and interference microscopy. The microscope is a modified Zeiss Photomicroscope I; the recorder is the 'Rectiriter' of the Texas Instruments Co.


1.0
0.5
0
-0.5
F = 10^4
CONTRAST
Δ0B = 0.01
Δ0B = 0.02
π-3.0
-2.0
-1.0
0
+1.0
+2.0
+3.0π
BACKGROUND PHASE RETARDATION ΔB (RADIANS)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CONTRAST
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Δ_{OB}=0.1
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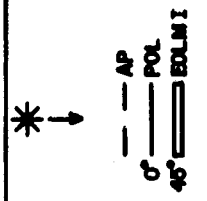
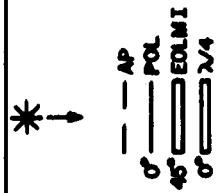
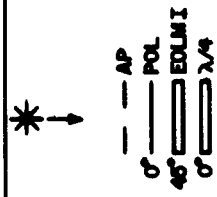
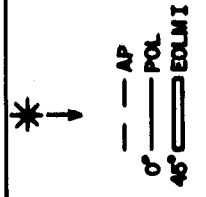
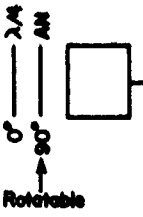
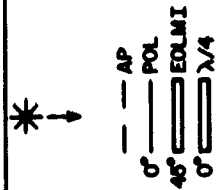
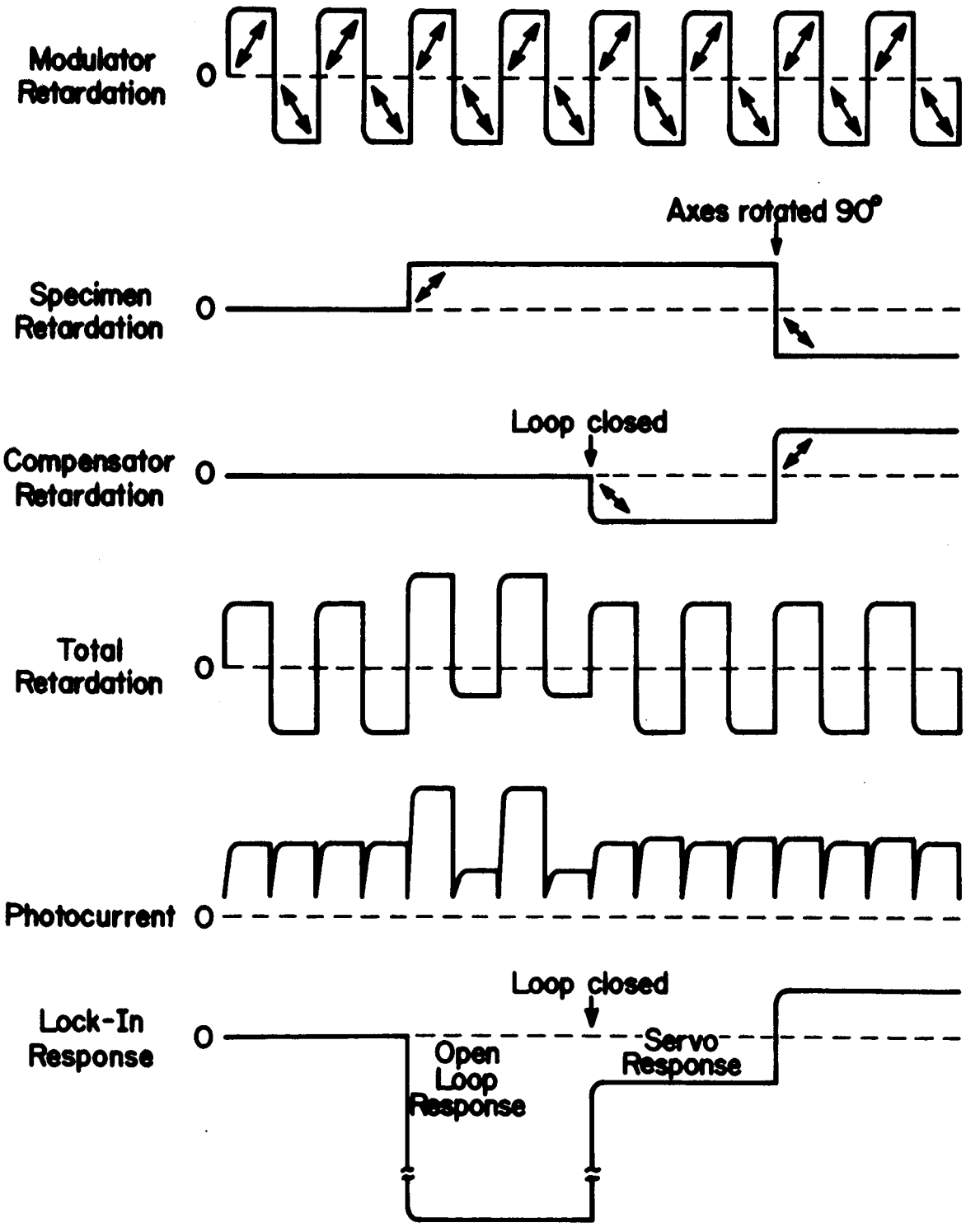
Δ_{OB}=0.5
π -3.0
-2.0
-1.0
0
+1.0
+2.0
+3.0 π
BACKGROUND PHASE RETARDATION Δ_B (RADIANS)

POLARIZED LIGHT and INTERFERENCE MICROSCOPY

OPTICAL PROPERTY	MODE	SOURCE	MODULATOR	MICROSCOPE	PHOTO-MULTIPLIER	OUTPUT
Phase Retardations Due to Birefringence	Servo			Strain - Free Optics		Lock-In Amplifier
Optical Rotation Alone	Servo			Strain - Free Optics		Lock-In Amplifier
Optical Rotation in Birefringent Specimens	2 Channels 2 Servos			Strain - Free Optics		Lock-In Amplifier
Phase Retardations Due to Refraction	Servo			Interference Optics		Lock-In Amplifier
Dichroism (Detection Only)	Open Loop			Strain - Free Optics Specimen Rotated		Lock-In Amplifier


Modulator Retardation
0
Axes rotated 90°
Specimen Retardation
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Loop closed
Compensator Retardation
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Total Retardation
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Photocurrent
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Loop closed
Lock-In Response
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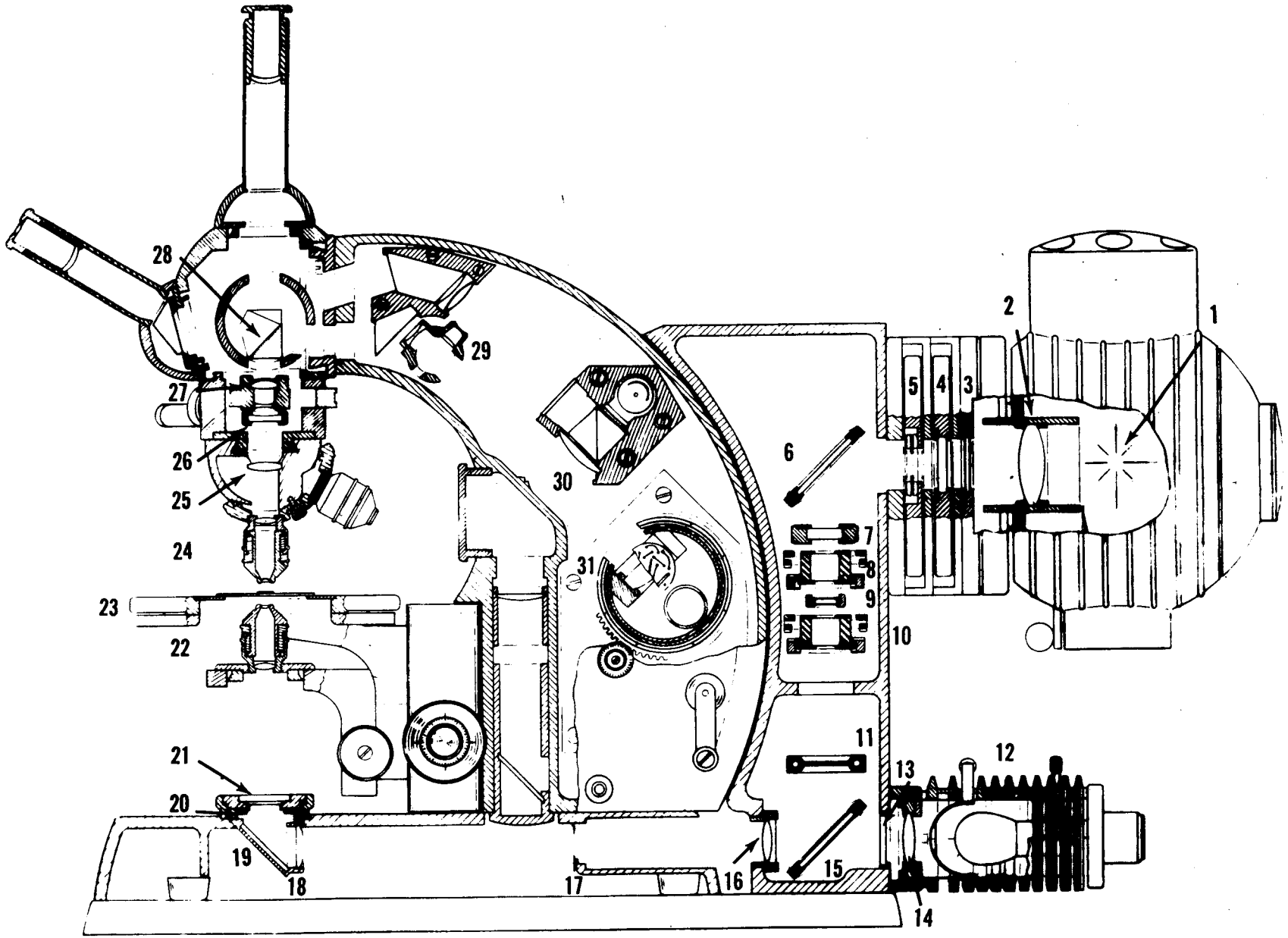
Open Loop Response
Servo Response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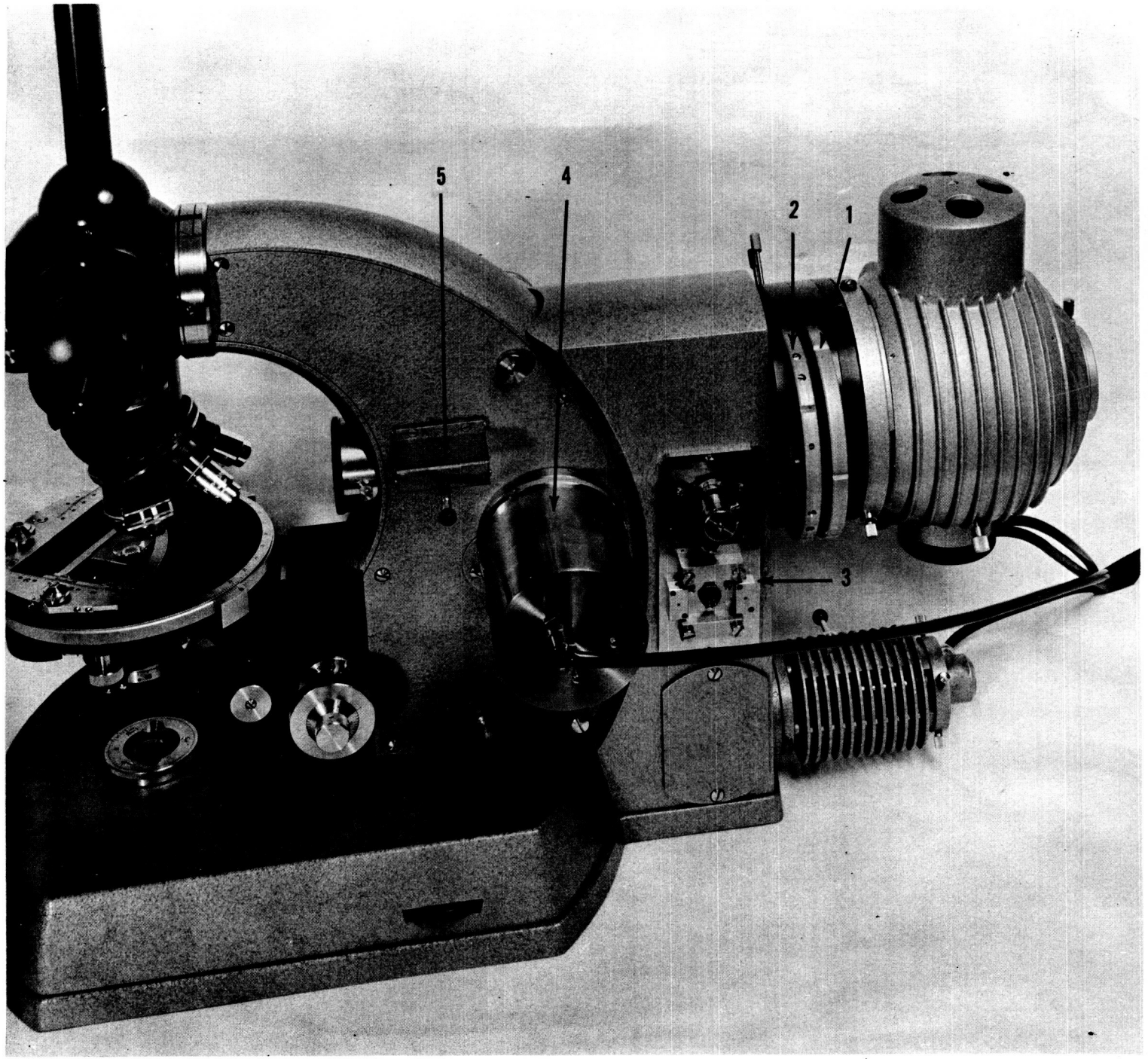
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2
1
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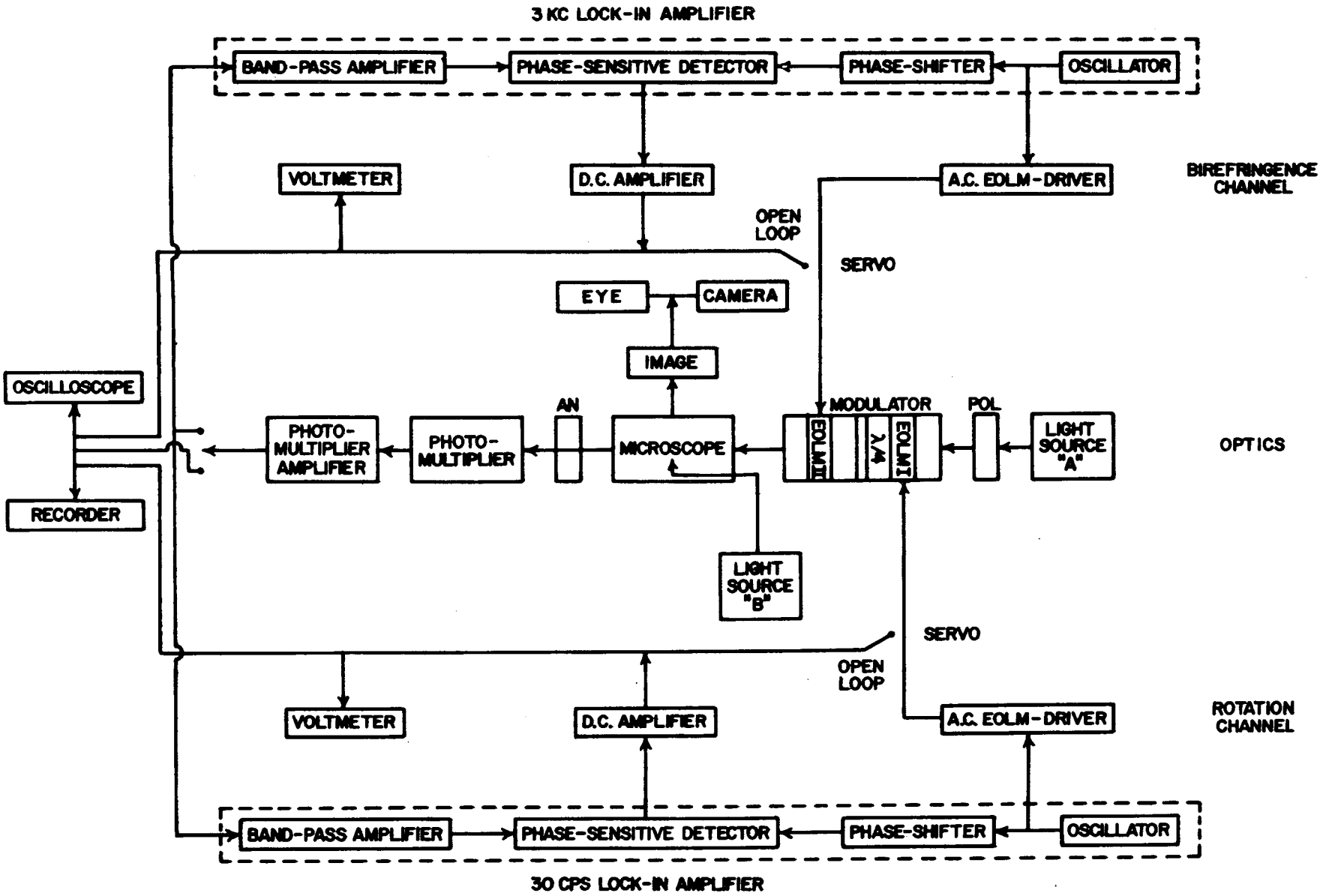
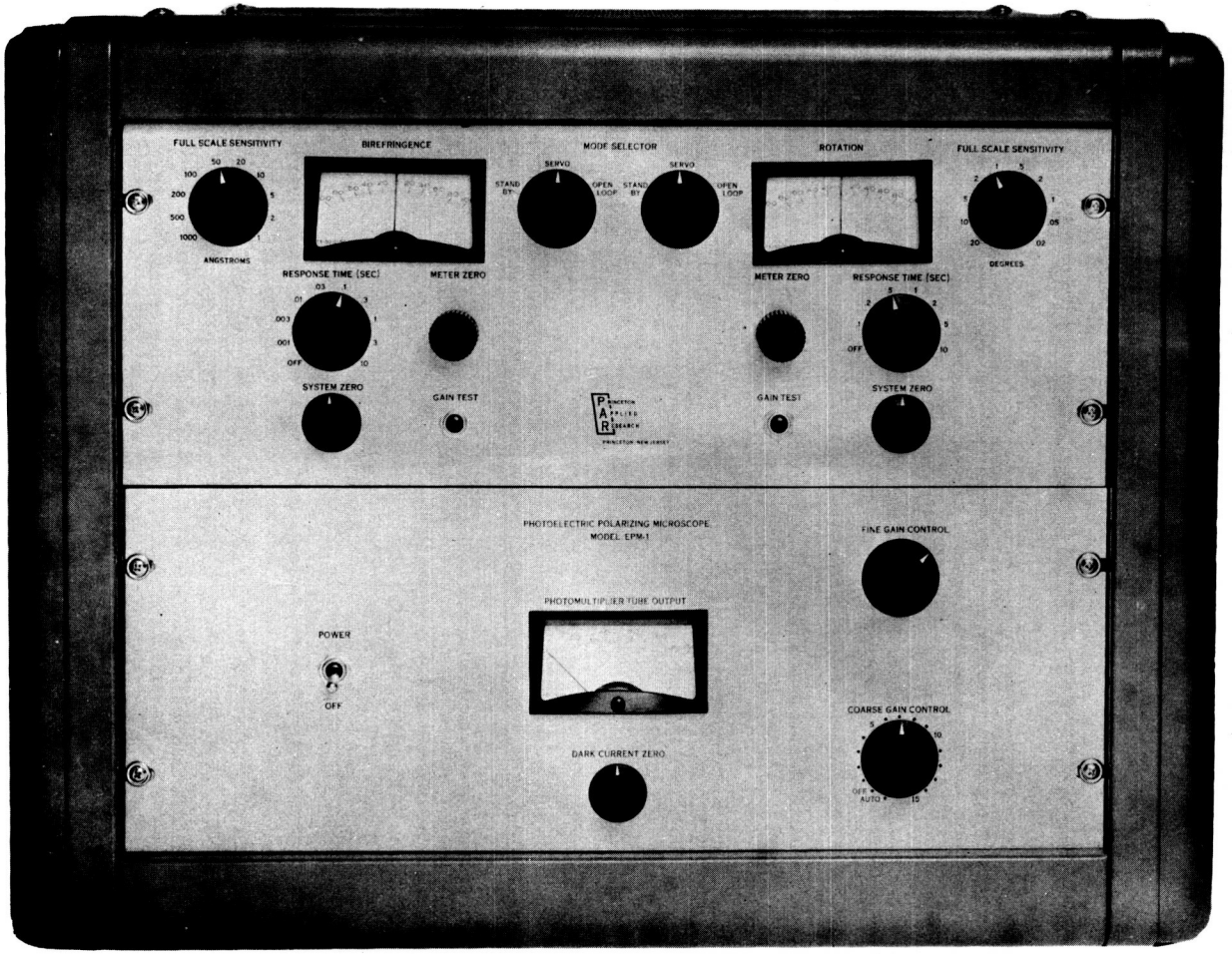
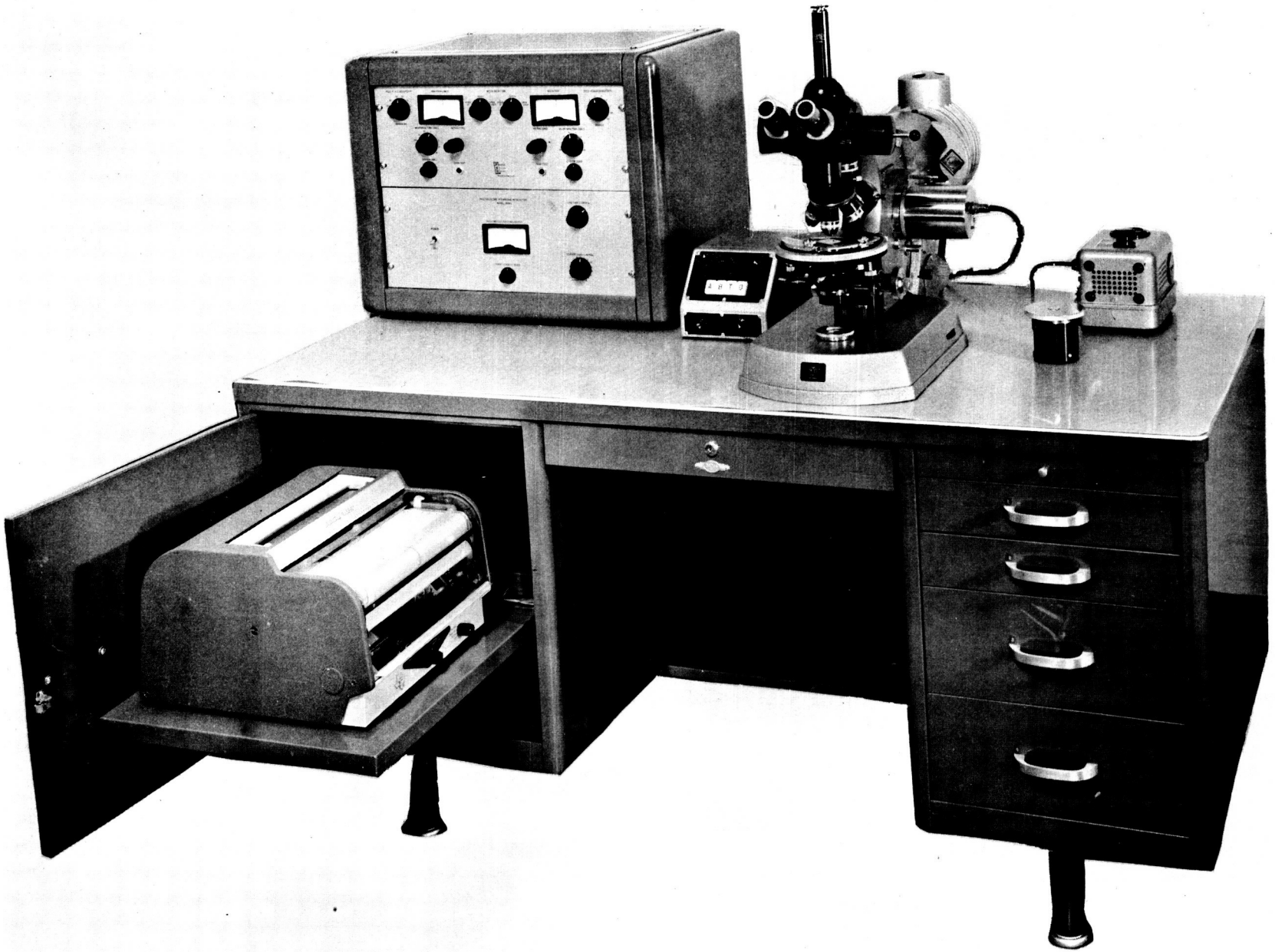

3 KC LOCK-IN AMPLIFIER
BAND-PASS AMPLIFIER
PHASE-SENSITIVE DETECTOR
PHASE-SHIFTER
OSCILLATOR
VOLTMETER
D.C. AMPLIFIER
A.C. EOLM-DRIVER
BIREFRINGENCE CHANNEL
OPEN LOOP
SERVO
EYE
CAMERA
IMAGE
OSCILLOSCOPE
AN
MODULATOR
POL
PHOTO-MULTIPLIER AMPLIFIER
PHOTO-MULTIPLIER
MICROSCOPE
EOLM II
λ/4
EOLM I
LIGHT SOURCE "A"
OPTICS
RECORDER
LIGHT SOURCE "B"
SERVO
OPEN LOOP
VOLTMETER
D.C. AMPLIFIER
A.C. EOLM-DRIVER
ROTATION CHANNEL
BAND-PASS AMPLIFIER
PHASE-SENSITIVE DETECTOR
PHASE-SHIFTER
OSCILLATOR
30 CPS LOCK-IN AMPLIFIER


FULL SCALE SENSITIVITY
BIREFRINGENCE
MODE SELECTOR
ROTATION
FULL SCALE SENSITIVITY
ANGSTROMS
DEGREES
RESPONSE TIME (SEC)
METER ZERO
SYSTEM ZERO
GAIN TEST
PHOTOELECTRIC POLARIZING MICROSCOPE
MODEL EPM-1
FINE GAIN CONTROL
PHOTOMULTIPLIER TUBE OUTPUT
POWER
OFF
COARSE GAIN CONTROL
DARK CURRENT ZERO



PART II

INSTRUCTION MANUAL

PHOTOELECTRIC POLARIZING AND INTERFERENCE MICROSCOPE

MODEL EPM-1

PRINCETON APPLIED RESEARCH CORPORATION

POST OFFICE BOX 565

PRINCETON, NEW JERSEY

CONTENTS

I. OPTICAL ALIGNMENT

The following steps will prepare the optical part of the instrument for the various types of measurements of which it is capable.

A. THE BIREFRINGENCE CHANNEL

1. Turn on the zirconium oxide arc lamp (1) (N.B.; numbers in parenthesis refer to the identification numbers in Figure 5 of Part I).

2. Adjust the beam-splitter selector slide (28) in the microscope tube head to the red position.

3. Adjust the polarizer (7) and analyzer (26) until the best deep red extinction cross is seen through the vertical microscope tube.

4. Insert the ocular and objective lens and center the objective on the center of rotation of the revolving stage.

5. Insert the selected inverted objective in the condenser mount and center it by bringing an image of the nearly closed rear diaphragm (17) into the plane of the specimen and center of the field.

6. Interpose the desired microbeam aperture into the light path by rotating the aperture selector drum (5). Be sure that special achromatic lens #1 (16) is in place, so that the first image of the microbeam aperture appears near the plane of (17). The rear (17) and front (20) diaphragms must be fully open.

7. Center the microbeam aperture with the two centering screws which protrude from the left of the aperture drum (5).

8. Rotate the filter drum (4) to select the desired interference filter (406, 437,546, and 576 millimicrons).

9. Pull out the slide to remove the camera photocell beam splitter (30) from the light path.

10. Set lens selector (29) for the lower magnification (3.2 X)

11. If desired, a quarter-wave plate may be inserted in the slot provided in the microscope tube head (between positions 25 and 26). (N.B.; This must be removed for optical rotation measurements.)

B. THE ROTATION CHANNEL

For optical rotation measurements, steps 1-10 are followed as before, and then the following additional steps:

12. A quarter-wave plate is added after either EOLM if only one EOLM is used. If both channels and both EOLMs are used, then the quarter-wave plate is placed between them in the slot provided (9).

13. If a quarter-wave plate was placed in the tube head slot (step 11) it must be removed.

C. THE INTERFERENCE ATTACHMENTS

For measurements with the interference attachments, no quarter-wave plate may precede the microscope proper. Steps 1-11 should be followed as above with the exceptions noted below.

14. The interference condensor and objective are substituted for the strain-free objectives in steps 4 and 5, and the directions supplied with the ZEISS interference attachments are followed to obtain a background phase retardation of zero (black) or one wavelength of green light (first order red color).

II. OPERATION OF THE ELECTRICAL SYSTEM

A. ROUTINE ADJUSTMENTS

1. Turn on the master switch and allow time for the temperature of the instrument to stabilize. Large variations in room temperature will cause changes in the strain birefringence of lenses and contribute to an undesirable degree of instrument drift.

2. Open the camera shutter to allow light into the photomultiplier housing, and adjust the "Coarse Gain Control" until the "Photomultiplier Tube Output" meter reads 70% (at some position other than "auto"). Close the camera shutter and adjust the "Dark Current Zero" until the meter reads zero. Reopen the shutter.

3. Connect the birefringence channel cable to the EOLM.

4. Turn the "Mode Selector" to "Servo," the "Full Scale Sensitivity" to 50 Angstroms, and adjust the meter to zero with the "Meter Zero" control.

5. For the time being, set the "System Zero" control to about the center of its range and the "Response Time" at 0.1 second.

6. Level the EOLM by adjusting two Allen-head screws located on either side of the EOLM connector. Leveling is performed while watching the meter by adjusting each Allen-head screw separately. The meter reading will reach a peak (the desired setting) and then decline. The second screw is adjusted to peak the meter in the opposite direction. If the adjustment range is insufficient, there are two coarse adjustment screws located inside the modulator housing on either side of the EOLM.

7. Adjust the "System Zero" until there is no change in meter reading when the "Mode Selector" is switched between "Servo" and "Open Loop."

8. The meter is then zeroed with the "Meter Zero" control.

9. Adjust the mirror phase control (11) until there is a minimum change in the position of the phase retardation meter when the analyzer is rotated ± 1 degree.

10. A rotatable collar in the photomultiplier housing (31) contains four apertures which may be used to limit stray light from the auxiliary illumination system. To select the proper one, first turn the "Coarse Gain Control" to OFF, then remove the photomultiplier housing. Rotate the collar to put one of the larger apertures in the light path. Measure the light intensity reaching the photomultiplier tube at some selected position of the "Coarse Gain Control."

Then, turn the latter off, remove the photomultiplier housing, and interpose the next smaller aperture in the light beam. Repeat this procedure until the aperture is small enough to affect the light intensity reading. The correct aperture to use is the one that does not limit the intensity of the modulated light from the microbeam, but does limit the amount of unmodulated light reaching the photomultiplier.

11. The incandescent lamp may now be turned on to provide red illumination for the field of view. Be sure that the red filter is in place after the field lens of the tungsten lamp (13).

The system is now ready to use, and the specimen may be inserted and a measurement made.

B. THE FUNCTION OF THE CONTROLS

1. The "Full Scale Sensitivity" control changes the sensitivity of the meter. Since the meter is not in the servo loop, neither it nor the control can affect the servo performance.

2. The "Frequency Response" control is a filter selector which simultaneously controls the frequency response of the panel meter and pen recorder.

3. The "Meter Zero" control allows any part of the meter range to be expanded to look for small changes, etc. Its proper initial setting is found in steps II.A. 7 and 8.

4. The "System Zero" control applies a bias retardation by a DC voltage applied to the EOLM within the servo loop. Its proper setting is given in Step II.A.7.

5. The red "Gain Test" buttons (one for each channel) may be depressed at any time to provide an accurate measure of the servo gain. Initially, the test button is calibrated to change the meter 100 Angstroms or 5 degrees when the "Mode Selector" is in the "Open Loop" position (See step II.C.). The ratio

between the deflections of the meter or recorder in open loop and servo is the servo gain.

$$\text{Gain} = \frac{\text{meter reading-open loop}}{\text{meter reading-servo}} = \frac{100}{x}$$

6. The "Mode Selector" switch has "Open Loop," "Servo," and "Stand By" positions. In the latter, no potential is applied to the EOLM. When not in use (e.g. between measurements), the switch should be left on that position to conserve the EOLMs.

7. The "Coarse Gain Control" will be used most frequently in the "Auto" position designed to minimize servo error by keeping the photocurrent at a constantly high level. In the other positions, the anode voltage is increased by steps of 100 volts except for the last two positions, which increase the amplifier gain by factors of 3 and 10 respectively.

8. The "Recorder Selector Switch" on the recorder itself allows the following selections:

Position 1 - pens are disconnected from the rest of the system.

Position 2 - records the output of both lock-in channels.

Position 3 - records the birefringence channel and the photomultiplier amplifier output simultaneously.

Position 4 - records the rotation channel and the photomultiplier amplifier output.

9. The "Recorder Drive Control" is also mounted on the front of the recorder and has four positions. In the "Off" position the pens still move, depending on the position of the "Recorder Selector Switch."

10. There is a "Paper Speed Control" located on the left front of the recorder with five speed ranges.

C. ELECTRONIC ALIGNMENT PROCEDURE

NOTICE: THE FOLLOWING CIRCUIT CONTROLS HAVE BEEN FACTORY ADJUSTED FOR PEAK

PERFORMANCE OF THE INSTRUMENT AND IT SHOULD NOT BE NECESSARY TO READJUST THESE CONTROLS UNLESS THERE IS A COMPONENT FAILURE.

1. The ±24 volt power supply is located in the lower chassis. It is the fourth board from the left and has only one adjustment (R812 on the schematic diagram) which is used to set the voltage on the -24 volt line at -24 volts. Connect an accurate D.C. voltmeter between pin 6 and the chassis for this reading.

2. The photomultiplier tube power supply board is second from the left on the lower chassis. It has only one adjustment (R222 on the schematic) to set the maximum tube voltage to 1100 volts. Connect an accurate D.C. voltmeter between pin 1 and the chassis. Shut off all light to the photomultiplier by closing the camera shutter. Rotate the "Coarse Gain Control" to position 15. The meter should read 1100 volts.

3. The +1050 volt power supply is located between the front panel and the main chassis, and has only adjustment (R10106 on the schematic diagram). In making this adjustment the lower chassis has to be disconnected and removed from the cabinet. (CAUTION: GREAT CARE SHOULD BE TAKEN WHEN ADJUSTING THIS POWER SUPPLY. DO NOT ATTEMPT TO CONNECT METER LEADS WITH THE POWER ON.) Connect an accurate voltmeter between the end of R1017 (510K) closest to the tube and chassis. The meter should read 1050 volts when the power is turned on.

4. The 3KC and 30cps oscillator and phase-shifter boards are basically similar and are aligned by the same procedure. The oscillator boards are located in the upper chassis, fourth from the left (30cps channel) and fifth from the left (3KC channel). There are five adjustments in this board. The upper-most one is the "Amplitude" adjustment R400, the second from the top is the "Frequency" adjustment R417, the third from the top is the "Phase" adjustment R438, the fourth from the top is the "Current" adjustment R414, and the last is the "Symmetry" adjustment R437.

a. The amplitude control is set by connecting a good oscilloscope between the base of Q407 (2N1304) and the chassis. The control is adjusted for maximum

signal amplitude without distortion of the sine wave.

b. The frequency control is found on the third board from the left (30 cps) and sixth from the left (3KC). Connect a good oscilloscope between pin 15 of the mixer board and ground. With the lamp on and the beam splitter set to allow light to enter the photomultiplier housing, turn the appropriate channel "Mode Selector" to"Open Loop." Adjust the "Coarse Gain Control" so that the photomultiplier amplifier meter reads about 70% of full scale. Place a quarter-wave plate on the microscope stage and rotate it until a sine wave appears on the oscilloscope. Adjust the frequency control for a sine wave of maximum amplitude.

c. The current adjustment is made with a good voltmeter connected across R327 (910 ohms). Turn the "Mode Selector" to "Servo" and adjust until the meter reads 10 volts.

d. The symmetry adjustment is made by connecting an oscilloscope across the output connector (CAUTION: 2000 VOLTS IS PRESENT AT THIS POINT). Adjust the symmetry control R437 until the square wave's positive and negative positions are of equal amplitude.

5. The voltmeters for the two channels are identical, and the following alignment procedure pertains to both. The voltmeter is located in the upper chassis, one at each end. There are two adjustments:

a. The common mode rejection control R1104 is located closest to the connector. Connect a 5 megohm resistor to connector pin #2. Connect another 5 megohm resistor to pin #3. The other end of both resistors is connected to the same output pin of an audio oscillator. The other end of the oscillator is grounded to the chassis. With the "Mode Selector" in "Stand By," adjust the "Full Scale Sensitivity" to one Angstrom unit. Zero the meter with the "Meter Zero" control on the front panel. Connect a good differential oscilloscope to the base of Q1102 (S865) and the base of Q1103 (S865). Adjust the oscillator for a 1 KC signal. The signal on the oscilloscope should reach a minimum when the common mode rejection control is set properly.

b. To adjust the meter calibration control, introduce a known phase retardation by rotating a quarter-wave plate on the stage (See Part I, 3C) or introduce a known optical rotation by rotating the analyzer. The meter calibration control should be adjusted until the recorder and meter agree with the standard.

6. The photomultiplier automatic gain control is located on the photomultiplier amplifier board, (lower chassis, first circuit board on the left). There is only one adjustment (R113 on the schematic). The purpose of the automatic gain control (the "Auto" position on the "Coarse Gain Control" adjustment) is to keep the gain of the servo constant when the sample density changes. It does this by controlling the voltage across the photomultiplier tube in such a manner that the output of the photomultiplier amplifier remains constant. Care should be taken when using the "Auto" position, as the photomultiplier amplifier cannot distinguish between birefringence signals, rotation signals, and noise. This control should be set for best servo performance and may require some trial and error before the best setting is found.

7. The gain test button is normally adjusted for an open loop change of 100 Angstroms or 5 degrees. The gain test may be calibrated in the following manner: Turn the "Mode Selector" to "Open Loop," "Microscope Beam Splitter (28) Selector" to "Red Position", shutter open, "Coarse Gain Control" set for optimum servo performance, "Full Scale Sensitivity" of 100 Angstroms (or 5 degrees), and the channel meter at zero. Depress the "Gain Test" button and adjust the gain test calibration potentiometer located on the rear panel until the meter reads full scale.