The Theory of the Microscope

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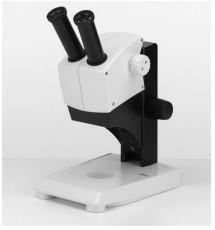

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The Theory of the Microscope

1.0 Introduction

The aim of this booklet is to provide the microscopist with a basic explanation of the theory of the microscope sufficient to enable him to understand the reasons behind accepted microscope techniques. It is felt that such an understanding will not only add to his interest in using the microscope, but will help him to work his way out of possible problems that may arise later on when detailed instructions, originally grasped, may have been forgotten. Where possible, mathematical formulae have been avoided, in favor of physical or pictorial explanations, as it is felt that such explanations are more easily grasped and better retained than explanations involving mathematics.

2.0 What's in a Microscope?

Figure 1 shows the way the lens elements of a microscope act to produce an enlarged image of a very tiny object. For the sake of clarity the 3 drawings are limited to strictly the lens elements of the microscope. Later on we will describe the complete system, including the illumination system, the substage condenser, and the mirrors and prisms in the binocular body.

At the right in Figure 1 is shown the traditional microscope. The objective acts much like a small projection lens, but instead of projecting an image onto a screen, it projects an enlarged primary image of the object up near the top of the microscope tube. This primary image is formed in the air and is called an "aerial image." (The presence of this image could be shown by removing the eyepiece and putting a small translucent screen in the plane of this aerial image.) In actual use, however, we do not use a screen, we look at this image through the eyepiece. This eyepiece acts very much like a magnifier, the principal difference being that it is used to magnify an aerial image instead of an actual object.

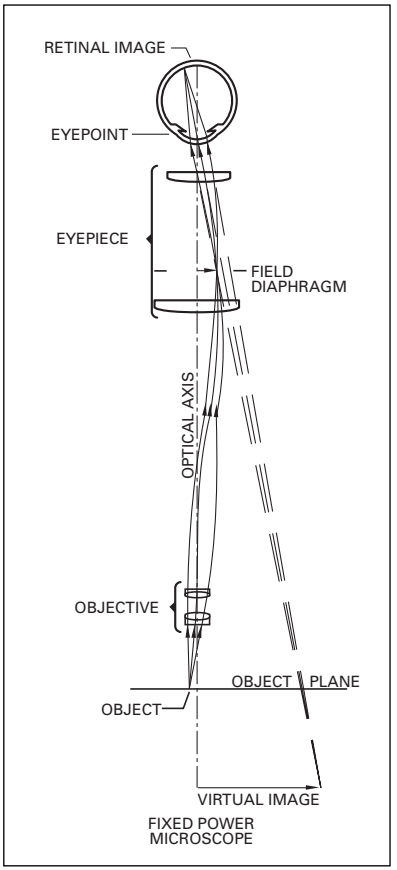

Figure 1

The final image is formed on the retina of the eye, but appears to the eye to be in the plane of the Virtual Image down near the bottom of the diagram. This latter image is called a "virtual image" because the light rays do not actually come from this image, they merely appear to come from it. The dashed lines going to the ends of this virtual image indicate that these are not actual rays of light, merely extensions of the actual rays. The actual rays are shown in solid lines in between the eyepiece and the eye point. It is instructive to lay a straightedge along the dashed lines to get a clearer picture of the fact that these are extensions of actual rays.

The microscope attains its magnification in two stages. The first stage of magnification is produced by the objective, the second by the eyepiece. The final magnification is the product of these two stages. If the objective magnification is 10 and the eyepiece magnification is 10, the final magnification is the product of the two, or 100.

3.0 What Limits the Magnification?

Actually there is no upward limit to the magnification of a microscope, there is only a limit to the useful magnification. The basic limitation is not magnification but resolving power, the ability of the microscope to render visible the fine detail of the object. If the object has been magnified to the point that its image is becoming fuzzy or indistinct, due to the limited resolving power, further magnification does nothing but make the image larger, and less distinct without showing any more detail. Such useless increase in magnification is called "empty magnification," meaning that it has exceeded the actual useful limit in showing specimen detail.

4.0 Resolving Power

The resolving power of a microscope depends generally on the design of the objective. An objective capable of utilizing a large angular cone of light coming from the specimen will have better resolving power than an objective limited to a smaller cone of light. This is shown in the comparison pictures, Figure 2. The objective which picks up the larger cone of light gives considerably more detail in the image.

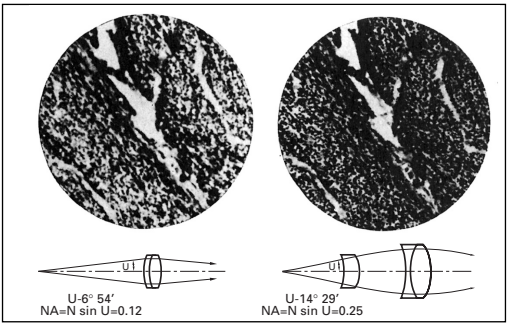

Figure 2
Photomicrographs showing the dependence of Resolving Power on numerical aperture (N.A.)

The image of a point object is not a point but, due to the phenomenon of diffraction, is a small circular spot of light surrounded by rings of light as shown in Figure 3. This phenomenon was first mathematically investigated by the astronomer Sir George Airy in 1834. He showed that the distribution of light in this pattern (which came to be known as the "Airy disc") is such that the radius of the first dark ring (h' in Figure 3) is a measure of the resolvable separation in the image. It can further be shown that this separation in the image can be referred back to the object as a separation h defined by the equation:

$$h = \frac{0.61\lambda}{N.A.}$$

where λ is the wavelength of light (about 0.0005mm or 0.00002") and N.A. is the "Numerical Aperture" of the objective.

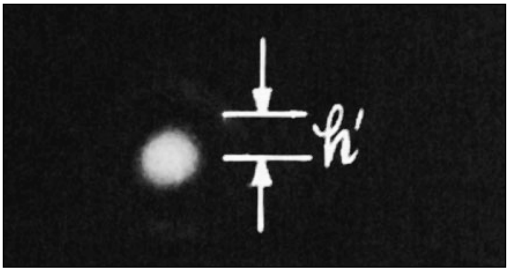

Figure 3
The Airy disc. Photomicrograph of a pin-hole in an aluminum mirror taken with a 4mm, 0.65 N.A. objective. h', the radius of the first dark ring, is a measure of the resolving power.

5.0 Numerical Aperture

The quantity N sin U in Figure 2 is called the Numerical Aperture or N.A. of an objective. Thus: N.A. = N sin U (by definition) where N is the refractive index in the object space.

Manufacturers customarily engrave the N.A. on an objective, as it is an important characteristic of the lens. The higher the N.A. the more complex and expensive the lens system becomes, hence in buying a microscope it is wise to see that the N.A.'s are up to standard practice.

$$\text{The equation } h = \frac{0.61\lambda}{N.A.}$$

indicates that the fineness of detail h which can be resolved is inversely proportional to the objective N.A.

As indicated by the formula above, there are three ways to increase resolving power, i.e. to decrease the resolvable separation h. The first method is to decrease the wavelength λ, the second is to increase the angle U in the object space, (see Figure 2), and the third is to increase the index N in the object space.

The wavelength λ can be decreased by going toward the violet, or short wavelength, end of the spectrum by means of selective filters. By means of special optics and special techniques this effect can be extended into the ultraviolet for still further lowering of the resolvable separation.

The angle U can be increased toward the 90° theoretical maximum, (i.e. N.A. = 1.00) only to a certain practical limit. The 0.95 N.A. apochromat represents a design giving the highest value of U which is practicable. N.A.'s higher than 0.95 are achieved by the use of immersion fluids, as explained in the next paragraph.

The final method of decreasing the resolvable separation is to increase N, the index in the object space. This is accomplished by "immersion objectives," in which a fluid is used between the object slide and the front lens of the objective. The immersion fluid is normally oil (N = 1.52) although water (N = 1.33) and monobromonapthalene (N = 1.66) have also been used to some extent. By means of immersion fluids, objectives as high as 1.60 N.A. have been produced, but the practicable limit has been found to be about 1.40 N.A.

6.0 Depth of Focus

When one focuses a microscope on an object, there is a finite range above and below this object in which other objects appear in sharp focus. This range is called the depth of focus of the microscope. It varies markedly with objective N.A., in accordance with the relation:

$$d = \frac{\lambda\sqrt{N^2 - (NA)^2}}{(NA)^2}$$

where *d* is the depth of focus for photomicrography. For visual use, one must add further depth, since the eye is capable of a certain amount of accommodation. In this case, the depth *d'* becomes

$$d' = d + \frac{250}{M^2}$$

where M is the magnification of the microscope. The assumption is made here that the eye can accommodate an image 250mm away.

Putting actual numbers in the above expression, we find that for the three most common objectives, we have the following depths of focus:

Objective	***Eyepiece***	***Depth of Focus***	
		Photomicro	*Visual*
10x, 0.25 N.A.	*10x*	*0.0080mm*	*0.0335mm*
40x, 0.65 N.A.	*10x*	*0.0010mm*	*0.0026mm*
100x, 1.25 N.A.	*10x*	*0.0003mm*	*0.0005mm*

The visual depth of focus of the 100X, 1.25 N.A. oil-immersion objective is here shown to be only 0.0005 mm, or about a wavelength of light. This very tiny value indicates how closely one must focus when using such a high power objective, and indicates why a microscope needs a very finely controlled focusing motion.

7.0 Image Aberrations

The perfect lens system has yet to be designed. Lens systems all have aberrations or defects to a greater or lesser extent, depending on the skill of the designer and the magnitude of the lens design problem. Lens systems are composed of lenses having spherical surfaces, and a spherical surface does not form a perfect image. The lens designer, by judicious combinations of lens shapes and glass choices, is generally able to counteract the defects of one surface by equal and opposite defects in other surfaces, so that the end result approaches perfection, even though it never fully reaches this goal.

The principal aberrations in the image formed by a spherical lens surface are:

I. Spherical Aberration

The (somewhat unfortunate) term used to express the fact that the outer portion of a spherical surface has more power than the inner portion. The lens designer overcomes this problem by judicious combinations of convergent and divergent lens elements, properly shaped to minimize the variation of focal power with aperture. Figure 4 shows the appearance of the Airy disc (image of a point object) in the presence of spherical aberration. Note, by comparison with Figure 3, that spherical aberration has caused some of the light which should be in the central spot to diffuse out into the ring structure. This undesirable diffusion causes a loss in contrast in the normal microscope preparation.

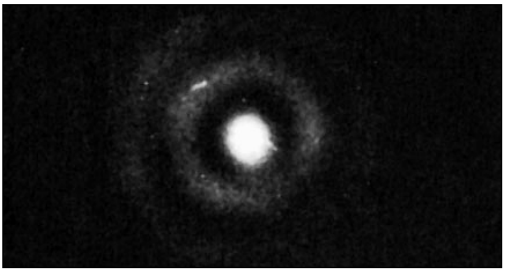

Figure 4
The appearance of the Airy disc when spherical aberration is present. Photomicrograph taken with a 4mm, 0.65 N.A. objective, purposely spaced incorrectly to show the effect of lens spacing on spherical aberration. Compare with Figure 3.

2. Astigmatism

is the defect whereby a marginal point object is drawn out into two separate line images lying at different distances from the lens surface. Like curvature of field, it results in a general deterioration of the off-axis image, but unlike curvature of field, an astigmatic image can never be

focused sharply except for the detail that is parallel or perpendicular to a radius of the field. Figure 5 shows the bad deterioration of the image of a point object (pinhole in an aluminum mirror) due to astigmatism.

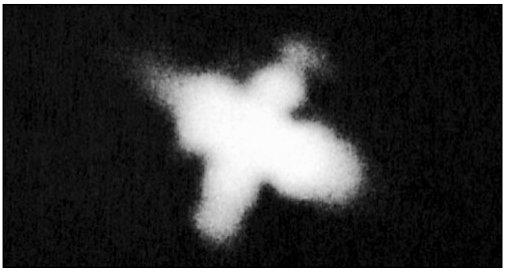

Figure 5
The appearance of a point object due to the presence of astigmatism. Compare with Figure 3.

3. Coma

is the name given to the defect in which different circular concentric zones of the lens surface give different magnifications to an off-axis image.

This defect results in a point object being imaged as a comet-shaped image, and, like the preceding two aberrations, causes the off-axis image to deteriorate. Figure 6 shows the bad deterioration of the image of a point object due to the presence of coma. Coma in the center of the field is an indication of damage to the objective.

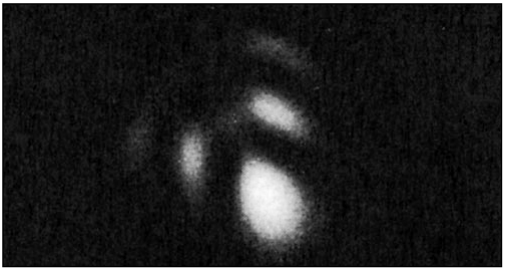

Figure 6
The appearance of a point object due to the presence of coma. Compare with Figure 3.

4. Distortion

is the aberration which renders a square object as an image with curved sides, as shown in Figure 7, an image of a rectilinear cross-ruling. Note how the rulings near the edge appear curved inward. This is called "pincushion distortion." The opposite effect is sometimes encountered, where the rulings appear to be curved outward, and in this case the effect would be termed "barrel distortion." Distortion is caused by the lens surface having different magnifications at the marginal and central portion of the image.

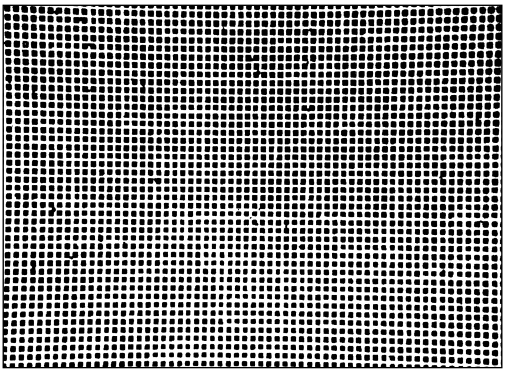

Figure 7
Distortion in a lens system causes straight lines to appear curved. Note curving lines at edges.

5. Curvature of Field,

as the name implies, is the aberration of a spherical lens surface which produces a curved image of a flat object due to the marginal portions of the image coming to a focus at a different distance than the central portions of the image. The end result is that when the central part of the image is focused sharply the marginal portions are out of focus, and vice versa. Figure 8 shows the appearance of the same cross-ruling as in Figure 7, but taken with a lens having curvature of field.

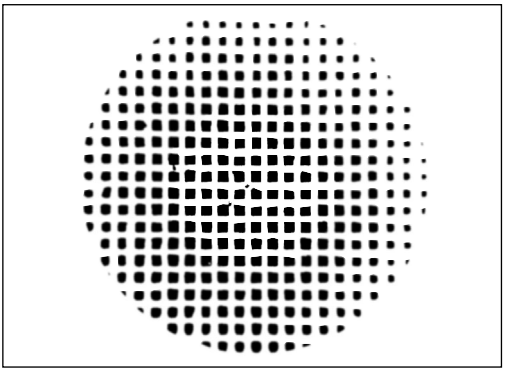

Figure 8
Curvature of Field. The center is sharply focused, the periphery is out of focus.

6. Chromatic Aberration

is the property of a spherical lens surface which brings light of short wavelength to a focus closer than light of a longer wavelength. The defect is brought under control by proper combinations of glass types used in the convergent and divergent lens elements which make up the lens system.

7. Lateral Color,

or chromatic difference of magnification, results in light of one color being imaged at a greater magnification than light of another color. This aberration causes an off-axis image of a point object to be spread out into a tiny spectrum or spread of color.

8.0 Aberrations in the Microscope

Of all the aberrations, spherical aberration is the one which the microscopist should understand and know how to control. It manifests itself as a loss in contrast, giving a hazy appearance to the image. Assuming you have a good quality microscope, the presence of spherical aberration usually indicates the use of the wrong cover glass thickness. The proper cover glass thickness is engraved on the barrel of the objective, and this thickness should be used for best results. This is particularly true of the high powered dry objectives. Objectives are normally designed for 0.17 mm thick cover glasses, and any appreciable divergence from this thickness causes spherical aberration, particularly with the high powered dry objectives, resulting in a "washed-out" image of low contrast.

Astigmatism

is normally present only to a minor degree in the off-axis image. If present off-axis, this is a design characteristic, and cannot be controlled by the microscopist. If present on-axis it is generally due to poor workmanship in the objective lens system.

The same remarks on off-axis astigmatism apply to off-axis coma. The presence of coma on axis, however, is caused by decentration of some of the lens elements, and could indicate the microscope has been mistreated.

Distortion

is normally under rather good control in a microscope. Some of the older negative lens systems used in photomicrography had considerable distortion, but even here, the types of objects generally viewed did not put very severe demands on the distortion correction, and such lenses were generally quite acceptable.

Curvature of field

has long been the most difficult aberration to contend with in microscope design. This was particularly true of the high powered objectives, where the extremely strong front elements left the designer with an almost hopeless task of correcting the field curvature. In the 1950's and 1960's various elaborate objectives having good flat field correction were introduced as premium priced items by several microscope manufacturers. 1965 ushered in a new era in microscope objective design with the introduction of flat-field objectives to replace the conventional achromatic series of objectives. In this new design concept, a 5X negative doublet lens system acts as a component of each of the objectives. (By taking over a portion of the magnification burden, this 5X system eased the magnification requirements on the objectives, and permitted the attainment of flat-fields without the extreme complexity and cost of prior systems.) Figure 9 shows the conventional achromatic series of objectives, standard for many years on all laboratory microscopes. Figure 10, for comparison, shows the newer flat-field objective series wherein the upper doublet is a common lens system for each of the objectives.

Note that in the higher powered flat-field objectives the front element is a thick meniscus lens, whereas in the older achromatic series the front element is a very strong hemisphere. This latter element was the principal cause of curvature of field in the older series.

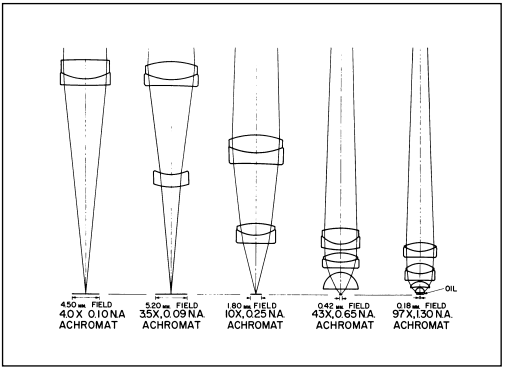

Figure 9
A series of Achromatic Objectives.

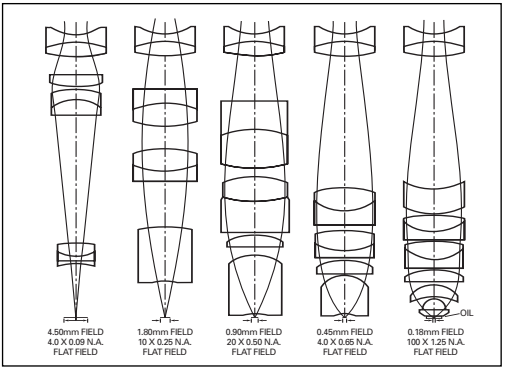

Figure 10
A series of Flat-Field Objectives.

Chromatic aberration

is normally not present to any appreciable degree in a well made microscope, whereas lateral color may often be found, particularly where the incorrect combination of objective and eyepiece is used. With the older achromatic series, Figure 9, the correction for lateral color was different from one objective to another, so that one usually compromised on lateral color in selecting an eyepiece for this series. With the new flat-field series, Figure 10, the degree of correction for lateral color is constant, and one eyepiece serves for the entire series. Note that the 4X objective in the flat-field series is more complex than its counterpart in the achromatic series. This is due to the design requirement that the entire series should have the same lateral color correction.

9.0 Apochromatic Objectives

A good many years ago it was found that an objective lens system which combined fluorite with glass lenses achieved an improvement over what was possible in the achromats, which were restricted to glass elements only. Such lenses became known as "fluorites" and "apochromats." The fluorites are sometimes called "semi-apochromats" as they represent a compromise between the achromat and apochromat in their degree of correction. Apochromats are superior to achromats in their correction for spherical and chromatic aberration. Also they are generally somewhat higher in N.A., hence have superior resolving power. Figure 11 shows a series of four apochromats, which may be compared with Figure 9. The shaded elements in Figure 11 represent lenses made of fluorite.

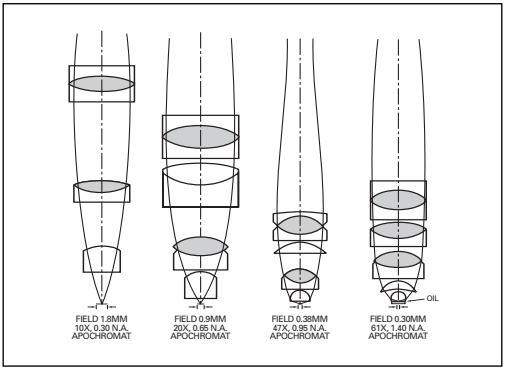

Figure 11
A series of Apochromatic Objectives.

10.0 Types of Eyepieces

As pointed out in the early sections of this booklet, the eyepiece is essentially a magnifier to enlarge the image formed by the objective. The least expensive and most widely used eyepiece is the Huygenian type, (Figure 12), named after its inventor, Christian Huygens, a famous Dutch scientist of the 17th century. The Huygenian eyepiece works well with the flat-field series and the low power achromats, but is afflicted with some lateral color when used with higher power achromats or apochromats. Its field of view is not large, and has a rather short eye-relief. Despite these shortcomings it finds wide acceptance because it is inexpensive and generally gives a very credible image.

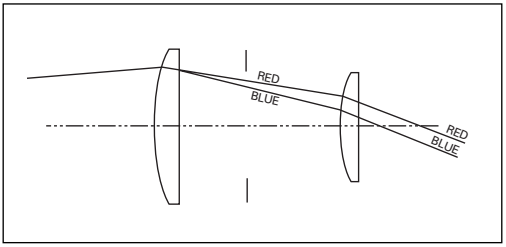

Figure 12
General construction of the Huygenian Eyepiece.

The Huygenian eyepiece utilizes two simple lenses to achieve its correction for lateral color. The light going through the first lens, called the "field-lens", is spread out into a spectrum with red at one limit and blue at the other. However, by proper spacing of the lenses, the red light is caused to strike the second lens, called the "eyelens" at a sufficiently greater height than the blue to compensate the angular spread between the rays and cause the red and blue rays to emerge parallel, as shown in Figure 12. To the eye, focused for infinity, the red and blue rays unite on the retina to form a color free image.

The widefield eyepiece, designed originally for stereomicroscopes, has subsequently been modified to work with the conventional laboratory microscopes. It is gradually supplanting Huygenian eyepieces because of its larger field of view and more comfortable eye-relief. Its lateral color correction is close to that of the Huygenian eyepiece, hence it works well with the flat-field objectives. A special version of this eyepiece is available for use with the zooming microscopes to get the optimum lateral color correction in this application. Figure 13 shows the two versions of the widefield eyepiece, one for the fixed power microscopes and the other for the zooming microscopes.

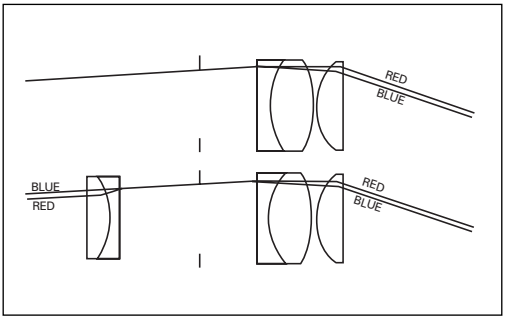

Figure 13
Two forms of Widefield eyepieces. The lower one has a lateral color correcting doublet added, for optimum performance with the Zooming Microscopes.

The Hyperplane and Compensating eyepieces, Figure 14, are similar in construction to the Huygenian eyepiece, except that the eyelens is a doublet. This construction gives more lateral color correction, as indicated in the figure. The compensating type gives the most correction for lateral color, the Hyperplane being a compromise between the Huygenian and the Compensating in this regard. Note that in Figure 14 the red and blue rays are shown aimed at different heights in the primary image, due to the objective having under-corrected lateral color. The eyepiece compensates for this, and causes the red and blue rays to emerge parallel so that they unite in a single color-free image on the retina of the eye. The higher power eyepieces in both the Hyperplane and Compensating series are of a more complex form than indicated in Figure 14.

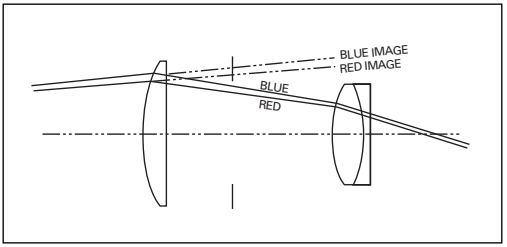

Figure 14
General construction of the low power Hyperplane and Compensating Eyepieces

In photomicrography special divergent (negative) lens systems are frequently used in place of the traditional eyepieces. In these systems there is no intermediate formation of an image as in an ordinary eyepiece. Instead, the image is intercepted before it comes to focus, and projected outward to the photographic film. Typical of such divergent lens systems are the Ultraplanes, available in three different degrees of lateral color compensation, for use with external cameras and for objectives ranging from low power achromats to high power apochromats. The modern built-in camera also utilizes this divergent lens concept. Here the system is corrected for use with the entire Flat-Field series of objectives. It is permanently aligned, and permanently parfocalized with the binocular viewing system, so that the binocular may be used as the focusing viewfinder for the camera.

11.0. Binocular Observation

Thus far we have considered only the monocular microscope. For prolonged use, the inclined binocular form is preferred since it gives a more natural and restful condition of observation. Figure 15 shows a cutaway view of the optical system of a binocular microscope. Binocular vision is attained by the use of a beam-dividing prism and three mirrors. This system divides the light equally, sending half to the left eye and half to the right. The coating on the mirrors is enhanced aluminum, having a multiple-film transparent coating on top of the aluminum to increase the reflectivity and protect the aluminum. Cover glass seals are used to keep dust from entering the binocular body.

12.0 The Illumination System

The illumination system is a very important part of the microscope. It is the part with which the operator can do most in controlling the microscope performance, and is at the same time probably the least understood part of the microscope. It has been pointed out in the preceding sections that the resolving power depends on the N.A. of the objective. To get the most out of the objective, the condenser system must be capable of delivering as large an angular cone of light as the objective is capable of utilizing.

This statement should not be interpreted as meaning that the full N.A. of the objective should always be illuminated. Generally speaking, full N.A. illumination is not used, because contrast drops as the illuminated N.A. approaches the full objective N.A. Each specimen is a law unto itself in regard to the proper illuminating N.A. With high contrast specimens having very fine detail one would want to use very close to full N.A. illumination, but with low contrast objects, the illuminated N.A. would have to be reduced to prevent complete “washing out” of the image due to low contrast.

The illuminated N.A. is controlled by means of the substage iris. It is instructive to remove the eyepiece and look at the back lens of the objective while opening and closing the substage iris. An image of the iris will be seen in focus close to the back lens of the objective. Full-aperture illumination occurs when the iris is opened up just enough to include the full aperture of the objective lens.

Closing down the illuminated N.A. excessively is a common fault with beginners in microscopy. The contrast gets very good by so doing, but at a loss in resolution. The loss in resolution is not so easy to notice as the gain in contrast. Excessive closing down of the illuminated N.A. also gives rise to image artifacts due to diffraction. The experienced microscopist learns to

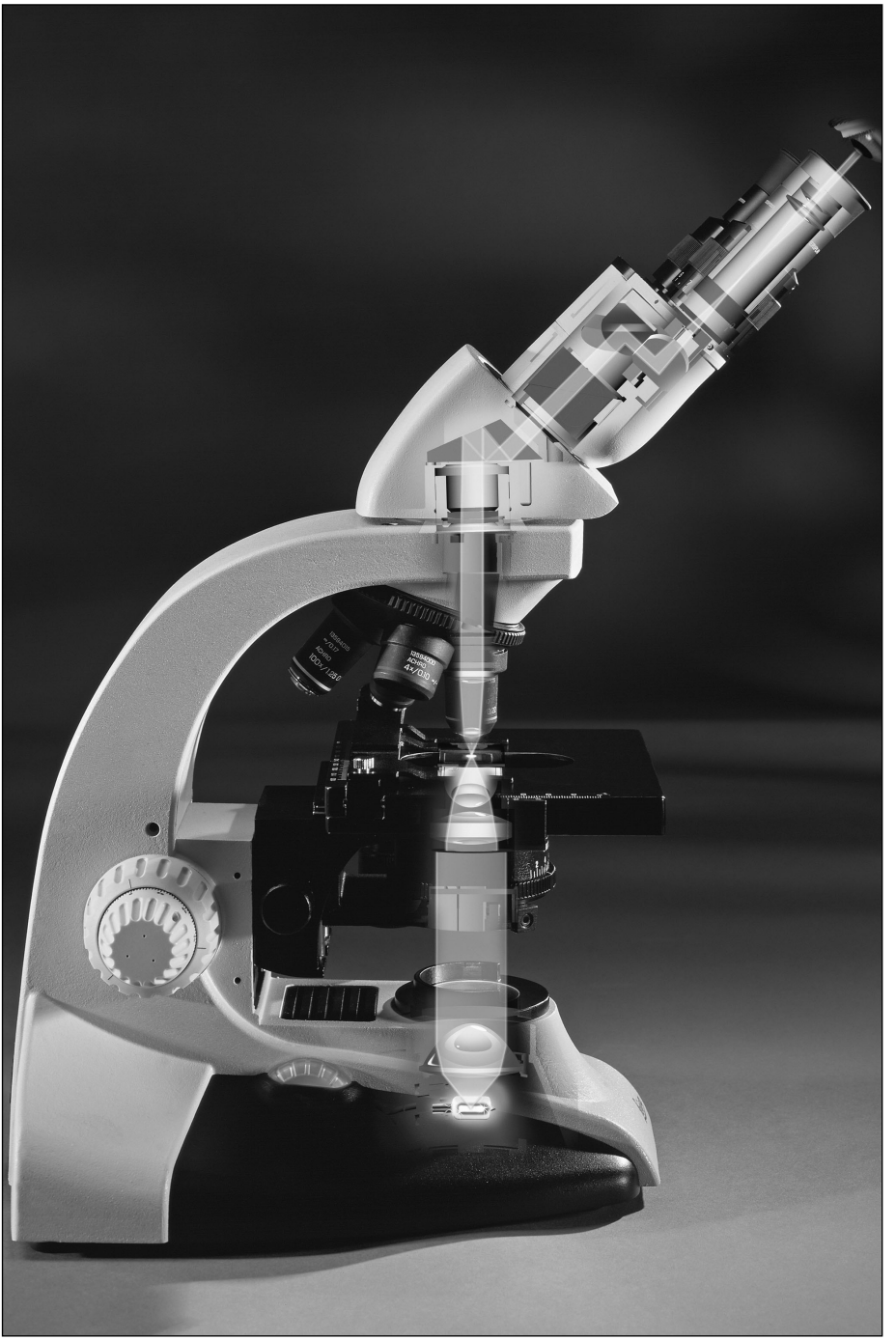

Figure 15

make the optimum setting for illuminated N.A. as a best compromise between resolving power and contrast.

Where the microscope illuminator has an iris diaphragm, it is intended to be used to control the size of the illuminated field of view on the specimen. The substage condenser should be adjusted to form a sharp image of the lamp iris on the specimen plane. The size of the iris should then be adjusted to lie just outside the field of view of the microscope.

13.0 Condenser Types

The function of the substage condenser is to direct a light beam of the desired N.A. and field size onto the specimen. There are several types of condensers, three of which are shown in Figure 16.

The Abbe Condenser is a 1.30 N.A. condenser utilizing only two lenses. Because of its simplicity and good light-gathering ability, it has become extensively used for general microscopy. It is, of course, not corrected for spherical or chromatic aberration, but for general visual observation it serves very well.

The Variable Focus Condenser is a two-lens condenser, 1.30 N.A. maximum in which the upper lens element is fixed and the lower one focusable. By this means it is possible to fill the field of low power objectives without the necessity of removing the top element when the lower lens is raised to its top position. This condenser is basically similar to the 1.30 N.A. Abbe. When the focusable lens is lowered, the focus of the light is brought in between the elements, and when this focus is at the point indicated in the diagram, the light emerges as a large diameter parallel bundle.

The Achromatic Condenser is a 1.40 N.A. condenser which is corrected for both chromatic and spherical aberrations. Because of its high degree of correction it is recommended for research microscopy and color photomicrography where the highest degree of perfection in the image is desired.

It will be recalled, from the previous discussion on immersion objectives, that to obtain N.A.'s over about 0.95 it is necessary to oil-contact the lens system to the specimen slide. A drop of immersion oil, placed on the lower surface of the object slide or the upper surface of the condenser hemisphere, achieves this result.

Generally it is not necessary to immerse the condenser, but special tasks occasionally do require immersion for optimum results.

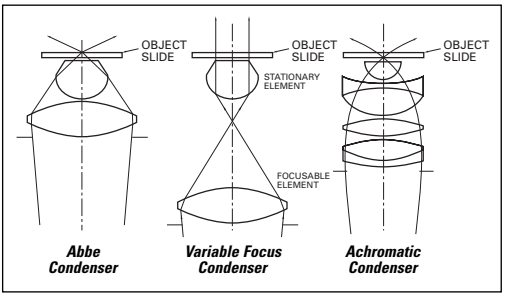

Figure 16
Condenser types.

14.0 Critical Illumination

Critical illumination is a form of illumination in which the light source is imaged directly on the specimen. It is used in high power microscopy, microprojection, and photomicrography, where an intense and controlled beam of light is necessary. Critical illumination was held in highest regard for many years, as early theoretical consideration indicated that it should permit higher resolving power than other forms of illumination. The reasoning for this was founded on the theoretical basis that two adjoining points in a specimen could be better resolved if their illuminated background had no point-to-point phase relationship. Such a background is provided by critical illumination where a source is imaged directly on the specimen, since the various points of a light source have the completely random phase distribution characteristic of thermal emission. With the passage of years, critical illumination has been gradually replaced by another intense form of illumination known as Koehler Illumination. The two systems are shown in Figure 17.

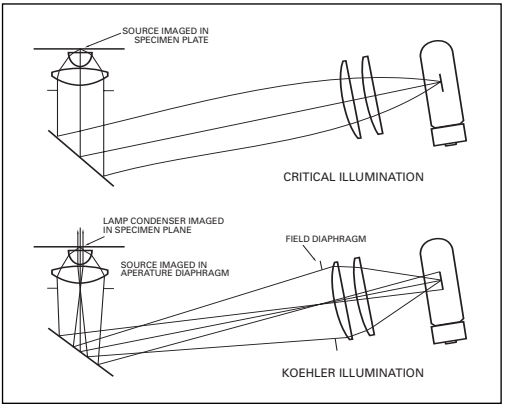

Figure 17
Illustrating both Critical and Koehler Illumination.

Koehler Illumination presents certain advantages over critical illumination and has gradually replaced the latter. With the Koehler system inclusion of field diaphragm control is made feasible. Another advantage of the Koehler system is that uneven distribution of energy in the source does not result in uneven brightness in the field of view, since the source is imaged in the aperture of the system. These practical advantages have led to the gradual replacement of critical illumination by Koehler Illumination, and when M. Berek showed that the two systems were theoretically equivalent in resolving power, the final argument favoring critical illumination was removed.

Many modern microscopes utilize built-in illumination systems. These are permanently aligned so that centering by the microscopist is not necessary. Usually they employ a low voltage concentrated filament lamp so that high brightness levels may be attained. Optically they are the equivalent of the Koehler system shown in the lower half of Figure 17, but one of the lamp condensers is invariably frosted to produce even illumination at all microscope powers, and the mirror is factory adjusted to give permanent centration to the illuminating beam.

15.0 Selective Filters

Contrast in the image of a colored microscope preparation can be controlled by the use of colored or selective filters. If, for example, the microscope preparation consists of red and blue areas, use of a red filter, which absorbs the blue but not the red, will darken the blue areas and cause the red areas to stand out bright by contrast with the blue. This type of contrast control is particularly useful in photomicrography and a set of selective filters is, accordingly, generally supplied with photomicrographic equipments. Colored glass filters and colored gelatine filters have been used extensively for this purpose in the past, but another type, operating on the principle of interference, has advantages over these, and will be described in the following section.

Interference Filters utilize the principle of optical interference to accomplish selective or colored transmission. The principle is illustrated in Figure 18. In the upper part of this figure, the effect of adding two waves which are in phase, is shown to result in a wave which is additive. The converse situation, where the waves are out of phase, is shown to result in destructive interference, or darkness. The former case is generally more easily understood than the latter. The "destruction" of energy by other energy is, however, understandable if labeled as a "redistribution" rather than a "destruction" of energy. The energy actually reappears elsewhere. For example, if destructive interference at a surface causes the transmitted energy to lessen, it will at the same time cause the reflected energy to increase. The interference filter utilizes this principle to accomplish conductive interference for a narrow band in the visual spectrum and destructive interference for the rest of the spectrum, so that only a narrow band of colored light is passed by the filter. The filter is composed of two semitransparent silver layers separated by

a thin layer of transparent material (magnesium fluoride generally). The thickness of the transparent layer is so controlled that multiple reflections between the silver layers are in a state of constructive interference in the transmitted beam for some chosen wavelength. The thickness chosen is slightly too great for constructive interference of shorter wavelengths, and slightly too small for longer wavelengths. As a consequence only wavelengths close to the desired wavelength get through the filter. The spectral transmission curve of a typical interference filter is shown in Figure 18.

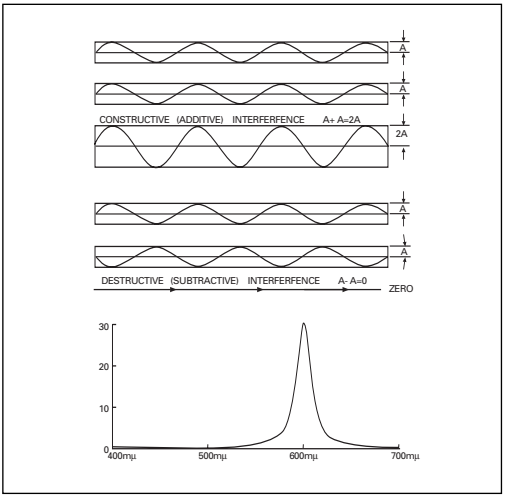

Figure 18
Interference Filters utilize the principle of optical interference to accomplish selective or colored transmission.

16.0 Darkfield Illumination

Two types of darkfield condensers are shown in Figure 19. These produce an intense hollow cone of light with apex (or focal point) in the plane of the specimen. If the specimen is completely transparent and homogeneous, the light continues directly on through and does not enter the objective, since the N.A. of the illuminating cone exceeds that of the objective. The field of view will thus look dark. If, however, the specimen has fine transparent detail which differs in refractive index from the embedding medium, it will scatter light due to refraction and reflection, and will appear bright, since some of this scattered light will enter the objective. This type of illumination, known as "darkfield," is useful principally on transparent unstained material where bright field illumination fails to make the object visible due to the low contrast. Figure 20 is a photomicrograph of such a specimen taken in darkfield illumination.

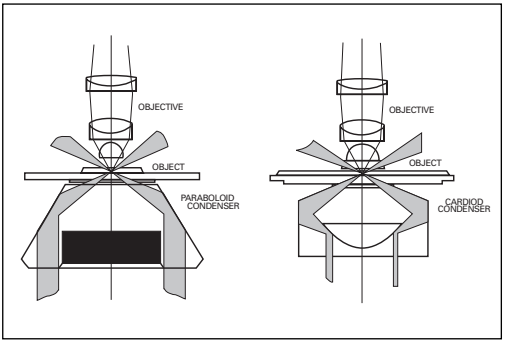

Figure 19
Two types of Darkfield Condensers. Each delivers a hollow intense cone of light, of greater N.A. than the objective N.A., hence objects are seen only by virtue of the light which they scatter.

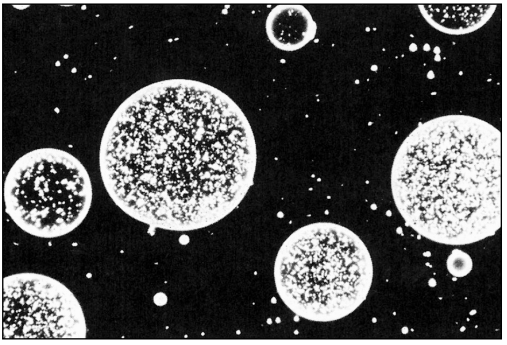

Figure 20
Emulsion of Sulphonated Oil in darkfield Photomicrograph by G.G. Schneider.

Darkfield condensers depend on the use of a high N.A. hollow cone of light, and must be oil-contacted to the lower face of the object slide in order to obtain the required N.A. in the illuminated cone. It is also, of course, necessary to use an objective of N.A. somewhat under the illuminated N.A. of the darkfield condenser, to avoid direct light getting into the image.

Objectives higher than 1.0 in N.A. must be provided with the appropriate "funnel stops" to reduce their N.A.'s to 1.0. The "funnel stop" is a small baffle, which fits into the back of an objective, reducing the aperture at the rear lens surface.

The effectiveness of a darkfield system is also dependent on the use of an intense, non-diffused, light beam from the lamp condenser. A homogeneous brilliant source, such as a ribbon filament or carbon arc, is required.

17.0 Polarized Light

Light energy is transmitted by waves, known as "transverse waves." This means simply that the waves vibrate at right angles to the direction of transmission of the light. Generally speaking, this vibration will be in any direction at right angles to the direction of transmission, but it is possible by means of devices known as "polarizers" to restrict the vibration to a single direction. If two such polarizers are inserted in the optical beam in such a manner that the second one transmits in a direction at right angles to the first, the light will be extinguished. Such an arrangement is called "crossed polarizers." If, however, in between these crossed polarizers we insert an object which is crystalline in nature, it will in general appear bright against the dark background caused by the crossed polarizers. Furthermore it will brighten and darken upon every 90° of rotation about the optical axis of the microscope. The reason for this is that crystalline materials generally have different properties in different directions, and as a consequence they alter the state of polarization of the light and thereby effectively "uncross" the polarizers. This form of illumination is particularly valuable in the study of crystalline chemical compounds and minerals. Very beautiful and striking color effects can be obtained by this method.

18.0 Fluorescence Illumination

A fluorescent specimen is one which when illuminated by light of one color, emits light of another color. Since fluorescence is usually rather weak it is necessary to use a very intense source, and to provide special filtering techniques to accentuate the fluorescent image.

The source used is normally a high pressure mercury arc, which has a number of strong emission lines in the spectral region between 300 and 600 nm. Most of the visible spectrum is filtered out by "exciter filters" which are located in the illuminating beam and pass only those wavelengths needed to excite fluorescence in the specimen. Since the deep blue and violet visible energy is not completely removed by the exciter filter, a second filter called a "barrier

filter" is used in the image forming system (i.e. after fluorescence has taken place) to remove the blue and violet but pass the longer fluorescent wavelength emitted by the specimen.

Another method of blocking off the unwanted blue and violet is to use dark-field illumination, so that no direct light of any wavelength can get through. Combination of the barrier filters with the dark-field system results in rather complete removal of the unwanted background of blue-violet light.

19.0 Phase Contrast Microscopy

The normal microscopic object is seen because it has regions of varying density. In normal "brightfield" illumination a completely transparent specimen is very difficult to see in any detail, as all parts are equally dense. Darkfield illumination shows up border effects in such completely transparent specimens due to edge scattering and diffraction. Polarized light is also useful when transparent specimens have directional or crystalline properties. Another form of illumination, known as phase contrast, is of value in the study of transparent media, and has found extensive use in the study of transparent living media where staining for normal illumination methods cannot be used.

Phase contrast is basically a method of illumination in which a portion of the light is treated differently from the rest, and subsequently caused to interfere with the rest, in such a manner as to produce a visible image of an otherwise invisible transparent specimen. The arrangement necessary for phase contrast is shown in Figure 21. A clear annulus in the focal plane of the condenser is imaged at infinity by the condenser and then reimaged by the objective in its rear focal plane. The undiffracted energy all passes through this image and is both reduced in intensity and given a quarter-wave phase shift with reference to the diffracted energy, by means of an annular phase pattern in the rear focal plane of the objective. The end effect of these two changes in the undiffracted portion of the beam is to simulate the phase and intensity distribution which would be present in the objective focal plane if the specimen had density variations rather than refractive index variations, and as a consequence the image formed by this beam interfering with the diffracted beam simulates that of a specimen having density variations.

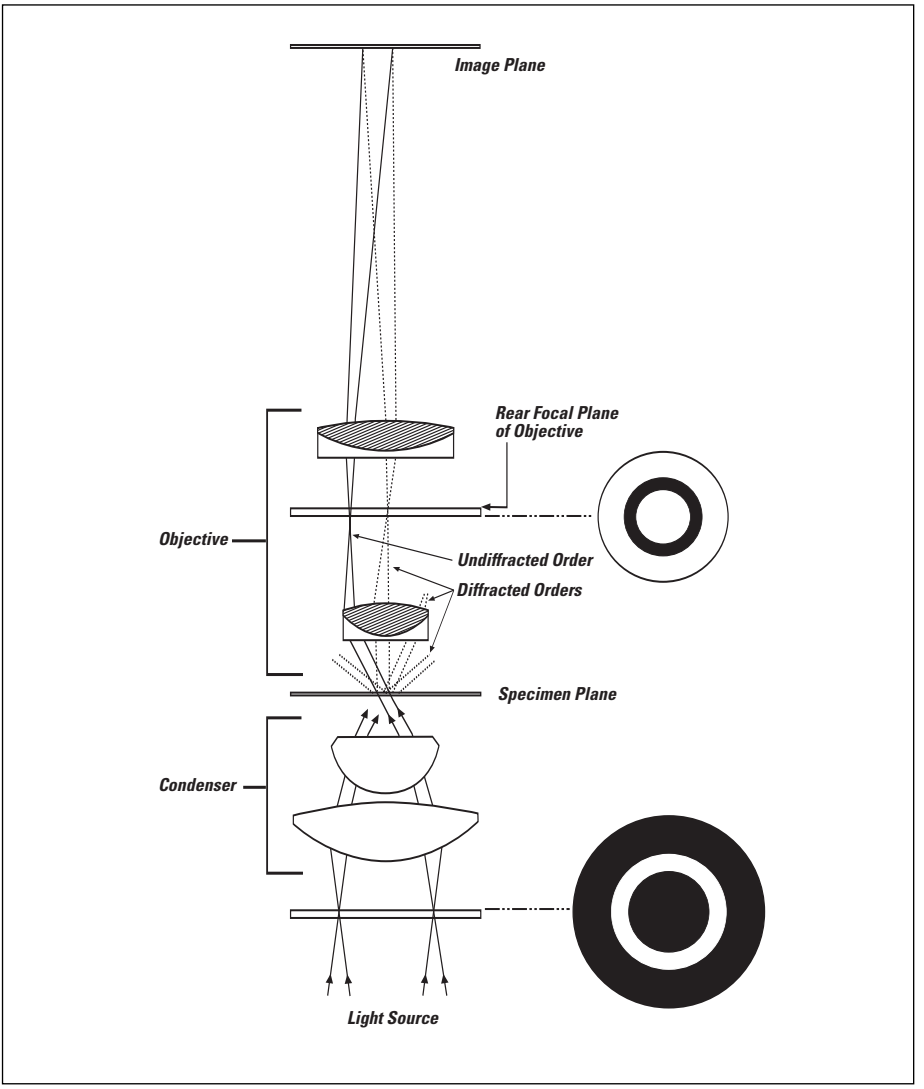

Figure 21

Image formation by Phase Contrast. An annular aperture in the diaphragm, placed in the focal plane of the substage condenser, controls the illumination on the object. The aperture is imaged by the condenser and objective at the rear focal plane, or exit pupil, of the objective. In this plane a phase shifting element, or phase plate, is placed. Light, shown by the solid lines and undeviated by the object structure, in passing through the phase altering pattern, acquires a one-quarter wave length of green light advance over that diffracted by the object structure (broken lines) and passing through that region of the phase plate not covered by the altering pattern. The resultant interference effects of the two portions of light form the final image. Altered phase relations in the illuminating rays, induced by otherwise invisible elements in the specimen, are translated into brightness differences by the phase altering plate. (The eyepiece is not shown in this diagram.)

20.0 Concluding Remarks

This booklet has aimed toward helping the microscopist understand some of the basic theory of the microscope. It is admittedly an abbreviated text on a subject which might well be extended to several times the length of this text. The aim has been to present the material in an interesting non-mathematical style, and it is recognized that in some cases this has oversimplified some of the explanations. The reader is encouraged to go on to more extensive treatments in the literature, and it is hoped that this booklet may have stimulated interest to do so.

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