COMPARATIVE MICROSCOPY

OF
BLEACHED, UNSTAINED SOFTWOOD TRACHEIDS
INCLUDING
MEASUREMENT OF REFRACTIVE INDEX

by

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ABSTRACT

Because of difficulty experienced with visual observation of bleached, unstained softwood tracheids, a study was initiated to determine the best microscopic system for observation of this type of material. A comparison was made between bright-field, dark-field, polarising, phase, electron, fluorescence and interference microscopic systems. In addition, the optical characteristics of each type of microscope were studied to determine the reasons for the differences in visual results. The study was expanded to include an evaluation of the interference microscope and its use in measuring refractive index of bleached wood pulp.

The interference microscope was found to give visual images superior to those obtained with any of the other systems examined and thus constitutes an important contribution to the practice of microscopy.

Inconclusive results were obtained in the evaluation of the system of measurement of refractive index by means of the interference microscope.
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Figures 1, 2 and 5 are reproduced from "The Baker Interference Microscope" by kind permission of the publishers, C. Baker of Holborn, Limited.
I. INTRODUCTION

When a specimen is examined microscopically, the final visible result is dependent, in large part, upon the effect of the specimen on the energy passing through it, reflected from its surface or emitted by it. The energy may be further modified by changes in wave length, intensity or velocity brought about by the instrument used. As the practice of microscopy has progressed, many methods have been introduced to improve the quality of the image obtained.

One general avenue of approach has been through the gradual improvement of the optical system. This system has evolved from the single lens of the earliest instruments to the compound microscope as we know it now, composed of many lenses, often of differing materials, and corrected to a high degree of optical perfection.

Concurrently with the technical improvement of the instrument, there has developed the art of histology which, by means of stains and other reagents, has sought to improve the visibility of microscopic sections and to learn something of their nature. Many of these stains are general in that they are used to increase the opacity of an entire specimen, increasing its effect upon the light it transmits and so differentiating it more clearly from its background. Others are specific stains which, by attaching themselves only to certain portions of a structure, render that portion more readily visible and also may indicate something of its nature. An example of the specific type of stain is Sudan IV,
which is used to identify fatty materials.

A third approach to the problem has been through the use of modified types of energy, either through the use of sources other than those producing white light, or through the use of devices which modify the form of energy used, either before or after it comes in contact with the specimen under observation. One reason for the developments which have taken place in this direction has undoubtedly been the fact that histological preparations, although they may give excellent visibility, are invariably fatal to living organisms. They do not permit of examinations of cells which are still carrying on the life processes.

An early modification of light employed was the technique of throwing the object out of focus and decreasing the substage aperture to a minimum. With nearly transparent objects, this had the effect of widening and darkening the details of the object, giving it more contrast with its background. This method has been superseded by methods which achieve the same improvement in contrast while allowing the specimen to be kept in focus.

The practice of colouring the illuminating light by means of filters, singly or in combination, has been made use of for a long time. The amount of diffraction through a given interface varies with the wavelength of the incident light. Light containing more than one wavelength, passing through a simple lens, forms a separate image for each wavelength and the images then occur in spatially separated planes. The use of monochromatic light produced by filters obviates this multiple image
formation. This type of aberration has been largely overcome through the development of achromatic optical systems which image light of mixed wavelengths in one plane.

Filters have also been used to produce colours complementary to those introduced by histological stains, modifying the effect of the stains in situ. Further advantages derived from using filters with bright-field microscopes have been the reduction of the amount of glare in the optical system and the adjustment of the quality of the light to ease the strain on the observer's eyes.

One of the first uses of instrumental modifications of the energy source (as distinct from changes in the image-enlarging system) was the introduction of the polarising microscope for examining objects by polarised light. This instrument, while excellent for certain purposes, is limited to objects with certain optical properties.

Many other specialised instruments have been developed. The dark-field microscope utilises the light-scattering property of some materials to make them self-illuminating. The fluorescence microscope makes use of the ability of certain substances to produce fluorescent light when illuminated by ultraviolet light. The development of fluorescent, histological stains has led to increased use of the latter microscope for examining specimens which do not themselves fluoresce.

Great progress has been made in increasing resolution by the introduction of the electron microscope. This instrument has made available much information not otherwise obtainable. However, for objects
of a scale which falls within the normal range of optical instruments, it has no advantages and many disadvantages.

The greatest advance in optical microscopy in recent times was undoubtedly the development by Zernike of the principle of the phase microscope. This made possible the production of good optical images without resorting to staining, thus permitting the critical study of otherwise difficult material, particularly living cells. This instrument converts optical path differences in the observed specimen into variations in the intensity of the transmitted light, rendering visible the details of structures which are transparent, or nearly so.

Optical path differences in an object change the "phase" of light passing through it. (See Page 9). Interest in this phenomenon of phase-changing, aroused by the work of Zernike, has led to the development of the interference microscope as a practical instrument for observation of many types of material. Various forms of interference microscope have been in use for many years in the study of the structure of surfaces. Interference microscopy, when applied to transparent objects, is, in effect, a more perfect form of phase microscopy. A method has now been developed for its application to such objects, providing superior images for visual observation and, at the same time, making it possible to obtain information about an observed specimen that is not easily obtainable by other means. The interference microscope represents a substantial contribution to the practice of microscopy.
II. OBJECTIVES

Dr. J. W. Wilson, while a member of the Faculty of Forestry at the University of British Columbia, did considerable experimental work in maceration, fiber length measurement and other fields that necessitated the frequent observation of softwood tracheids under the microscope. In the course of this work, a great deal of difficulty was experienced in studying the structure of these fibers because most of them were in a highly bleached condition due to the maceration techniques employed. Histological staining was used to increase the quality of the microscopic images formed. However, it was very difficult to obtain satisfactory visual images with many batches of macerated material simply because the material would not take stain well. Even when the staining was adequate, the techniques used were time-consuming.

It was decided, therefore, to make a comparative study of those methods of microscopy for which the instruments were available, together with such adaptations and refinements as might be devised, in order to determine the most suitable method for observing unstained, bleached softwood tracheids.

While this work was in progress, an interference microscope, made by C. Baker and Co. Ltd., of London was obtained on a loan basis. In addition to its normal use as an instrument for visual observation, this microscope was said, by the manufacturer, to be capable of measuring refractive index with great sensitivity. The scope of the study was increased to include an assessment of the value of this additional attribute for the study of softwood pulp at various stages in its manufacture.
III. SYSTEMS OF MICROSCOPY AS APPLIED TO
UNSTAINED BLEACHED SOFTWOOD TRACHEIDS

DEFINITION OF TERMS

Terminology is discussed here to avoid the insertion of explanations subsequently at times that might be inappropriate with respect to continuity of presentation.

1. Light Waves

Since the passage of light through a microscope is essentially a problem in refraction, it is customary to consider the light as progressing in the form of sinusoidal waves. Along the waves electric and magnetic fields exist which are directed at right angles to the direction of propagation and which periodically rise and fall in intensity. For present purposes it is permissible to ignore the magnetic component and consider one wave vibrating sinusoidally in one plane about an axis of propagation. Figure 1 illustrates three such waves travelling from left to right in the plane of the paper.

2. Wave Length

The distance between two adjacent points on a wave that are the same distance from the axis of propagation and are vibrating in the same direction is, by convention, called one wave length. (Fig.1, Wave A). Light travels at a constant velocity in a homogeneous medium. The period of vibration may vary for different waves. The wave length is determined by dividing the velocity by the period of vibration. Each wave length produces a different colour of light.
3. Amplitude and Intensity

The amplitude of a wave is the vertical distance of the wave from the axis of propagation at its moment of greatest departure from that axis. The intensity of a wave, which is perceived by the eye as relative brightness, is proportional to the square of the amplitude.

4. Phase

When two waves are vibrating along the same axis in the same plane but intersect the axis at spatially separated points, they are said to have a phase difference. This difference is expressed as a fraction of the wave length.

In Figure I, waves A and B are drawn separately for the sake of clarity. If they are superposed on the same axis in the same plane they will have a phase difference of one-eighth of a wave length.

5. Interference

The amplitudes of two waves vibrating in the same plane are algebraically additive at each moment of travel along the axis of propagation and give the effect of a single wave. Amplitudes above the axis are considered to be positive and those below the axis to be negative. This phenomenon is known as interference.

If the two waves have no phase difference, their amplitudes are complementary. Since intensity is proportional to the square of the amplitude, the apparent intensity is greater than the sum of the intensities of the individual values (27).

Where a phase difference exists, the resulting amplitude will depend upon the amplitudes and directions of travel of the two interfering waves at each instant of travel. The resultant amplitude (and
Figure 1

Wave diagram illustrating the combination of two waves with the same amplitude and a phase difference of $\frac{1}{8}$ wavelength.

Figure 2

Vector diagram illustrating the combination of two waves with the same amplitude and a phase difference of $\frac{1}{8}$ wavelength.
hence intensity) can range from zero to the maximum possible for the conditions existing.

A particular case of importance is when two interfering waves have equal amplitudes and wavelengths but a phase difference of one-half of a wavelength. Then their amplitudes will be exactly in opposition, will destroy each other, and zero amplitude, as well as zero intensity, will result. Visually, the result will be darkness.

6. Resolving Power

The resolving power of a microscope is the ability of the instrument to form a perfect point to point reproduction of the object in the image. It is usually expressed as the diameter of the smallest particle which can be made to form a discrete image.

7. Refractive Index

The refractive index of a substance expresses the relationship between the speed of light in the substance and the speed of light in a vacuum, the speed of light in a vacuum being set at unity (30). If the refractive index of a given liquid is 1.53, then the speed of light in the liquid is $1/1.53$ of the speed of light in a vacuum.

Refractive index is considered to be a function of the molecular structure of the substance (15).

8. Becke Line Test

When an interface composed of two substances of unequal refractive index is observed in the field of a microscope objective, a bright line, produced by refraction, is seen along the boundary between the two media. As the distance from the object to the objective is in-
creased, the bright line moves into the substance with higher refractive index. As this distance is decreased, the bright line moves into the substance with the lower refractive index (2).

By using an immersion medium of known refractive index, it is then possible to determine whether the object being examined microscopically has a higher or a lower refractive index. Immersion in successive media of different refractive indices will allow the bracketing of the value of the refractive index of the unknown. By continuing the process, the actual value of the unknown refractive index can eventually be determined with an accuracy dependent upon the tolerance within which the indices of the immersion media are known. Although time-consuming, the method is simple and precise (19).

9. Optical Path

The optical path through a substance is an expression of the effect of the substance upon the speed of light passing through it. The refractive index governs the relative speed of light through the substance in general and the thickness of a given section of the substance will determine the length of time the refractive index acts upon the light. The product of the thickness times the refractive index will give the optical path (30).

COMPARISON OF SYSTEMS OF MICROSCOPY

The gradual growth of the science of microscopy has led to the development of many different types of instruments. Many of these have been used in the past for studying the structure of the tracheids
of softwoods and excellent results have been obtained (13) (19) (7). However, where satisfactory results have been reported, the material under observation has usually been histologically stained or otherwise treated to improve its suitability as a microscopic specimen. The present study was initiated to determine if any of the available systems of microscopy could be used to produce a satisfactory image of bleached tracheids that were not stained. All tracheids used for comparison were of Douglas fir (Pseudotsuga menziesii (Mirb.) Franco), macerated by heating at 60°C in a mixture of 50 per cent glacial acetic acid and 50 per cent of 30 per cent hydrogen peroxide. The resulting pulp was in a highly bleached condition. The variety of microscopes used was determined by the types which were available.

The results obtained, together with an appreciation of two types of microscope that were not used, are set out below. In addition, an explanation is given as to why each type of microscope gave the results it did.

1. Bright-field Microscope

The human eye is sensitive to only two of the properties of the light entering it in the form of an image of an observed object—intensity and colour. The light reaching the eyepiece of a microscope can be modified by an object with respect to intensity, wavelength (colour) and phase. This light consists of two parts, that which passes through the object and forms the image of the object, and that which passes around the object and forms the background. Each of the two
parts has its own wavelength and intensity and, as well, the two may differ in phase. The light emanating from the substage condenser of a microscope may be considered as an aggregate of point sources of light. The light from each point source spreads over the whole field, illuminating the object and the background. If some rays from a point source are put out of phase by the object with other rays from the same point source which illuminate the background, the two sets of rays will interfere with a consequent change in the apparent intensity. In addition the object may change the intensity of the transmitted light by simple absorption.

If the object absorbs sufficient of the light to materially change the intensity, or changes the phase of the object light relative to the background light so as to cause destructive interference, the object will be darkened and appear in contrast with the background. The more uniformly transparent an object is, the less will be the change in intensity and the less the contrast between its components and between the object and the background.

Bleached, unstained tracheids did not give a satisfactory image when examined microscopically (Plates I, IV, VII, X, XV, XVIII). The material did not absorb enough of the transmitted light. It is also apparent that the optical path differences were not such as to cause phase changes sufficient to materially change the intensity of the light through interference. Definition was poor due to lack of contrast.

By the use of proper technique, particularly the use of papers of high contrast, good images can be recorded photographically
for tracheids in the field of a bright-field microscope. This however
is of no value as a visual aid. The illustrative plates were prepared
in such a way as to obtain an image as close as possible to that actu­
ally seen through the microscope.

If the substage iris diaphragm of a bright-field microscope
is reduced to an aperture much smaller than normal for the objective in
use, and, in addition, the object is thrown slightly out of focus, a
great deal more contrast between the object and the background can be
obtained. This is illustrated in Plates XIII and XIV. However, the
resolution suffers a great deal and the technique is not to be recommen­
ded when better methods are available.

2. Dark-field Microscope

A dark-field microscope differs from a bright-field microscope
in having an opaque disc inserted between the substage mirror and the
lower lens of the condenser. The disc is complete except for a narrow
annulus cut through it which permits only a narrow circle of light to
pass into the condenser. The condenser is constructed or adjusted to
have a very short focus. The result is the production of a hollow cone
of light which can be brought to a focus at the object but then passes
around the objective without entering it (Figure 3).

Light which is refracted by the object will be diverted from
the hollow cone and a large portion of it will enter the objective,
passing to the eyepiece as an image of the diffracting object.

If, as is usually the case, the object differs in refractive
index from the surrounding immersion medium, one or more refracting in­
terfaces will exist and light will be diverted to the objective. The
Figure 3

Diagrammatic representation of the path of light through a dark-field microscope.
sub-microscopic structure of cellulose consists in part of regularly arranged crystalline structures (1) (8) (10) (11) (19) (23) (25) (26). These have light-scattering powers greatly in excess of that due simply to the presence of interfaces between the cellulose and the surrounding medium. Bleached tracheids consist mainly of crystalline cellulose and are therefore strongly refringent and form a self-illuminated image in a dark-field microscope (Plates III, VI, IX, XII).

In theory, the greater the difference in refractive index between an object and its surrounding medium, the greater the refraction due to interfaces should be. In practice, the best images of tracheids were formed when immersed in tetrahydrofurfural alcohol which has a refractive index very close to that of the tracheids (6). No satisfactory explanation has yet been worked out for this.

Although bright images of bleached tracheids have been obtained with a dark-field microscope, all parts of the material are not equally refringent and the image is unevenly illuminated. As a result the resolution suffers particularly at high magnifications (Plate XII). Better resolution, and hence better images, have been obtained with other methods, as shown in subsequent sections.

3. Fluorescence Microscope

If an object having the property of fluorescence is illuminated with ultraviolet light, it emits light of wavelengths within the range visible to the human eye.

Ultraviolet light is absorbed in large measure by many types of optical glass. A fluorescence microscope must have a substage optical system that does not appreciably absorb this type of energy. A reflect-
ing condenser system may be used or the lenses of the condenser may be formed of a suitable material, such as quartz. Such microscopes are specialized and not readily available.

Bleached tracheids were not examined through a fluorescence microscope. However, an ultraviolet light was available and tracheids, illuminated with incident ultraviolet light, did not exhibit fluorescence to a degree detectable under a dissecting microscope. This line of enquiry was abandoned.

4. Electron Microscope

The development of the electron microscope has made possible the production of images with good resolution at magnifications many times greater than those usable with any form of optical microscope. Many examples are available showing the submicroscopic structure of tracheid walls (1) (8) (11) (13). However, there are many disadvantages attendant upon the use of this instrument. The field is extremely small, covering only a very small portion of a single tracheid. The specimens must be extremely thin to prevent excessive absorption of the electron beam. The technique required to produce images is time-consuming (25) (28).

The electron microscope, at its present stage of development, is not suitable for the examination of the structure of entire tracheids without the use of special techniques.

5. Polarising Microscope

Light of any wavelength, as generated, consists of vibrations in many planes. Certain prisms or films have the property of absorbing
all of these vibrations except those which vibrate in a certain plane. Such substances are said to plane polarise the light and are called polarisers.

A polarising microscope has one prism inserted below the condenser and another inserted between the objective and the eyepiece. Light passing through the lower polariser will be plane polarised in one direction of vibration. If the upper prism, or analyser, is rotated until its plane of polarisation is at right angles to that of the lower prism, no light will be transmitted through it.

All anisotropic crystals have one or more optic axes. An optic axis is a direction in the crystal in which light travels through the crystal without phase change or refraction. Light which passes through a crystal not parallel to an optic axis is doubly refracted into two component waves which are plane polarised at right angles to each other and travel in parallel paths. In addition, one wave is retarded behind the other, the amount of retardation increasing as the deviation from the optic axis increases. Since the frequency of both waves of light remains unaltered and the velocity changes proportionately, the relative wave length must change. The two waves, upon emerging, will be out of phase and interference will result.

A uniaxial crystal is one with only a single optic axis. If such a crystal is placed on the stage of a polarising microscope with its optic axis in the plane of vibration of the lower prism, the plane polarised light will pass through without phase change or refraction. If the upper prism is placed so its plane of vibration is at right angles to that of the lower prism, the unchanged, plane
polarised light will be absorbed. No image will be visible.

If the uniaxial crystal is rotated until its optic axis is no longer parallel to the plane of polarisation of the lower prism, the light will be resolved into two components vibrating in mutually perpendicular planes. Both components, upon passing through the upper prism are double refracted, but only the light vibrating in the plane of polarisation of the upper prism passes through. The phase difference impressed upon the waves by the uniaxial crystal will cause interference (27).

White light consists of many wavelengths, each wavelength producing a different colour. For small retardations, interference of waves will have only a small illuminative effect and the resulting wavelengths will combine to produce greys and white. A crystal causing a small retardation will appear grey and white (30).

Cellulose is composed, in large part, of parallel uniaxial crystals (8). A bleached tracheid observed under a polarising microscope will appear as a bright grey image when rotated so the plane polarised light from the lower prism strikes at the maximum angle to the optic axes of the crystals (Plate XVI). If the tracheid is rotated until the optic axes are parallel to the polarised light, an image will be produced only by those elements where the structure is diverted from the long axis of the tracheid (Plate XVII, XIX). From this much has been learned about the crystalline nature of cellulose (20).

As shown above, plane polarised light passing through a uniaxial crystal, not in the direction of the optic axis, is re-
fracted into two waves vibrating in mutually perpendicular planes and with a phase difference. If a second uniaxial crystal is placed in the path of the waves from the first crystal so that its optic axis is in the plane of vibration of one of the waves, the second wave will vibrate at right angles to the direction of the optic axis of the second crystal. One wave will be essentially unaffected but the velocity of the second will be changed. The second crystal can be set to increase the phase difference between the two entering waves. With the resulting higher retardation values, certain specific wavelengths will be eliminated, resulting in combination of other wavelengths to give the brilliant colours seen in Plate XX.

Good images of bleached, unstained tracheids were observed (Plates XVI, XIX). These, from a visual standpoint, were inferior to those obtained with the interference microscope for reasons which will be given in the section on that instrument.

6. Phase Microscope

The contrast in an image produced by absorption is not great when the object observed is uniformly transparent, or nearly so. Also the phase difference between light passing through the object and light forming the background, is usually not sufficient to cause much change in intensity through interference. For these reasons, relatively transparent objects, including bleached, unstained tracheids, do not form satisfactory images in a bright-field microscope. If however, the relative intensity of the object and background light can be changed, by artificially changing the phase difference or the absorption of the light, the quality of the image can be greatly improved. The phase microscope was built to incorporate these principles.
In a phase microscope, the light from the substage mirror, or other source of illumination, passes through a narrow annulus placed at the first focal plane of the condenser. The condenser is of normal focal length and the hollow cone of light produced passes into the objective and is imaged as a bright circle at the rear focal plane of the objective (Figure 4). Light refracted by the object is bent out of the hollow illuminating cone, passes into the objective, and is spread over the whole of the back focal plane of the objective. While there is a small amount of overlap in the area of the bright annulus, essentially the object light and the background light are effectively separated and can be treated individually (3).

If a plate is placed at the back focal plane of the objective, it will show a bright image of the annulus. This image will be surrounded by the light from the object, or will enclose this light. By etching the plate where it images the annulus, the optical path of the background light is changed. This increases the phase difference between the object and background illumination, resulting in greater interference and stronger contrast.

Many other modifications are possible, such as reducing the intensity of the background light by coating the plate with absorbing material in the region of the annulus image, but the essential principle remains the same.

Although bleached tracheids are a difficult subject because of their relative transparency, excellent visual images can be formed with the phase microscope (Plates II, V, VIII, XI).
Figure 1
Diagrammatic representation of the path of light through a phase microscope.
Because the separation of light is dependent upon the
diffracting power of the object, very small phase changes will not
diffract sufficient light to be differentiated in the final image.
This difficulty is overcome in the interference microscope. For
this reason phase microscopy is considered an imperfect form of
interference microscopy.

7. Interference Microscope

In a phase microscope, the mutually interfering pairs of
waves, which cause the contrast, are produced by diffraction in the
object. The process is limited by the inability of slightly re-
fracting portions of the object to bend the light sufficiently to
put it outside the hollow cone of illumination.

In an interference microscope, on the other hand, the
mutually interfering pairs of waves are generated by the instrument
independently of the object. The phase relationship of these trains
is modified by optical path differences between the object and the
surrounding medium.

Various types of interference microscopes are now being
marketed - instruments which differ in the means by which the inter-
fering waves are produced. The instrument available for this study
utilises polarised light and double refracting elements. A means is
also provided for varying the phase relationship between mutually
interfering waves.

Since the phase change brought about by an object varies
slightly for each wavelength of light, and the small phase change
produced by the object is further enhanced by the instrument, the image produced in white light appears coloured. By modifying the phase relationship, the colours can be moved from point to point in the object, permitting various portions to be brought into the best contrast for viewing.

When bleached, unstained tracheids are examined, the tracheid wall appears as one colour, the bordered pits appear as gradations of that colour, and the pit apertures appear as a different colour.

The colour contrast can be intensified by increasing the difference between the refractive index of the object and of the immersion medium. By using media of very high or low refractive indices, the cellulose wall shows a range of colours, each small difference in optical path being shown.

If monochromatic light is used, the object can be made to appear dark against a bright background or bright against a dark background by simple rotation of a polaroid plate. This change in phase difference is obtained in phase microscopy only by changing phase plates.

8. Optical System of the Baker Interference Microscope

The Baker interference microscope consists of a high quality bright-field microscope, with the addition of an interferometer system which is readily brought into, or removed from, the optical system. The optical system is shown diagrammatically in Figure 5.
Figure 5

Diagrammatic representation of the path of light through a Baker interference microscope.
Light from the substage mirror passes through the polarising plate and a plane polarised wave emerges. This passes through an iris diaphragm into the Abbe condenser. A double-refracting plate is cemented to the front of this condenser. The plate consists of a portion of a uniaxial crystal so the single wave emerges as two, one of which is displaced by refraction. The two waves are polarised in mutually perpendicular planes. One ray passes through the object and the other through the surrounding immersion medium. Because of optical path differences between the object and the medium, the two waves are no longer in phase and will interfere. A second double-refracting plate, exactly complementary to the first in refractive power, is cemented to the front of the objective. The two waves are recombined but the phase difference remains and the two waves still vibrate in mutually perpendicular planes.

Since the two waves are spatially separated when they leave the condenser plate, all parts of the object will cause interference, and very small phase differences will be effective in modifying the light.

The pair of interfering waves, after leaving the objective, pass through the quarter-wave plate. This plate, with the rotatable analyser forms a de Senarmont compensator which is capable of modifying the phase relation between the two waves (19). When white light is used, this phase changing system causes the shifting of colours. In monochromatic light it is the basis of the determination of refractive index as will subsequently be shown.
9. Quality of Image in the Baker Interference Microscope

The visual images of bleached, unstained tracheids were superior to those produced by any of the other systems tried. An observed tracheid appeared as a varicoloured image against a different coloured background. The image was comparable to that obtained with differential histological staining but was superior in two respects. The contrast with the background was improved because of the phase changing effect. And the colour of each component was variable at will so any portion could be brought into highest contrast.

The disadvantages inherent in a relatively transparent image in bright-field were overcome. The resolution and contrast were superior to those obtained with dark-field. Contrast was superior to that obtained with phase because the system is not limited by small changes in optical path and the contrast obtained was variable without interchange of attachments.

A polarising microscope can be used to produce colour contrast similar to that shown by the interference microscope (Plate XX). There is a somewhat comparable phase changing system available for a polarising microscope which consists of a quarter-wave plate which can be inserted into the optical system. Because the interference observed in a polarising microscope is dependent upon diffraction by the object, the range over which phase change will produce visible difference is reduced. Modulated colour contrast is not possible without the further modification of a rotatable analyser. Then the polarising microscope would function as an interference microscope to a limited extent, and
only with crystalline material of the proper type as the object.

Although the illustrations and discussion of comparative microscopy have been concerned with the results obtained with bleached, unstained tracheids, comparable results were obtained with other materials which were examined. The polarising and dark-field microscopes, of course, gave poorer results than those shown, for material with weaker diffracting properties than the tracheids.

The interference microscope was on temporary loan and had been returned to the owner before it was decided that photographic illustrations would be desirable. It was therefore not possible to present pictorially the images obtained with interference. Plate XX, taken with a polarising microscope, modified as described above, gives an approximation of these images. The actual images obtained with the Baker interference microscope showed a much greater gradation of colour.
Plate I. Bright-field. Douglas fir tracheid in triethylene glycol. (100 X).

Plate II. Phase Contrast. Same tracheid as Plate I in triethylene glycol. (100 X).
Plate III  Dark-field. Same tracheid as Plate I in triethylene glycol.  (100 X).

Plate IV  Bright-field. Douglas fir tracheid in water.  (200 X).
Plate V  Phase Contrast. Same tracheid as Plate IV in water. (200 X).

Plate VI  Dark-field. Same tracheid as Plate IV in water. (100 X).
Plate VII  Bright-field. Douglas fir tracheid in tetrahydrofurfural alcohol. (100 X).

Plate VIII  Phase Contrast. Same tracheid as Plate VII in tetrahydrofurfural alcohol. (100 X).
Plate IX Dark-field. Same tracheid as Plate VII in tetrahydrofurfural alcohol. (100X).

Plate X Bright-field. Douglas fir tracheid in tetrahydrofurfural alcohol. (200X).
Plate XI  Phase Contrast. Same tracheid as Plate X. 
In tetrahydrofurfural alcohol. (200 X).

Plate XII  Dark-field. Same tracheid as Plate X. 
In tetrahydrofurfural alcohol. (200 X).
Plate XIII  Bright-field. Douglas fir tracheid in water. Slightly out of focus. (100 X).

Plate XIV  Bright-field. As Plate XIII but substage aperture stopped down. (100 X).
Plate XV  Bright-field. Douglas fir tracheid in water. (100 X).

Plate XVI  Polarised light. Same tracheid as Plate XV between crossed nicol prisms. In water (100 X).
Plate XVII  Polarised Light. As Plate XVI but rotated 45° to extinction. In water. (100 X).

Plate XVIII  Bright-field. Douglas fir tracheid in water. (450 X)
Plate XIX  Polarised Light. Same tracheid as Plate XVIII between crossed nicol prisms. In Water. (450 X).

Plate XX  Polarised Light. Portion of Douglas fir tracheid showing colour contrast. For full explanation see text. In Water. (100 X).
IV MEASUREMENT OF REFRACTIVE INDICES
OF WOOD PULP

The interferometer system built into the Baker instrument, through measurement of phase change, can be used to determine various attributes of unstained, transparent material. Among these are the shape, volume and protein content of living cells (15) (2). The refractive index of an object can be found by either of two methods. Firstly, if the thickness of the object is known, or can be measured, the phase change brought about by the object with relation to an immersion medium of any known refractive index is measured by means of the analyser incorporated in the microscope. Knowing the thickness and the phase change, the refractive index of the object may be calculated. This is amplified below.

Secondly, if the thickness of the object is not known or cannot be measured, but the object can be transferred from one to another of two media of different refractive indices, the phase change caused with respect to each of the two media can be measured. The refractive index of the object may then be calculated.

Since the immersion media can have any known refractive indices, the first one can be a highly volatile substance. Then the object can be changed from one medium to another simply by immersing it in the first medium, taking the phase measurement and letting the medium evaporate. The second medium can then be introduced.

The first technique is applicable to substances where the thickness is known or to cylindrical fibres where the width can be
measured by an eyepiece micrometer (2). Tracheids vary greatly in the ratio between their various dimensions so the thickness cannot be determined by measuring the width as seen in the microscope. Measurement of thickness, by focusing with the microscope, is subject to considerable error (11) (12) and cannot be used with confidence where precision is required. For these reasons it was decided to use the method of immersion in two media for the current study.

THE SYSTEM OF MEASUREMENT

The intensity which results from the combination of the two waves originating from the same point on a luminous source will depend upon the individual intensities of the waves and also upon the distance between the crest of one wave and the corresponding crest of the other. By convention this is known as the phase difference between the waves.

Such phase difference can be represented by conventional vector diagrams, with the amplitude of each wave represented by the length of a vector and the distance between the crests of the two waves shown as the angle between the vectors, with one wavelength represented by 360° (Figure 2). Using the parallelogram rule for vectors, where the two waves have the same amplitude, the resultant amplitude (hence intensity) of the two waves is equal to twice the amplitude times the cosine of one-half the phase angle. The relative amplitude only is required, so the calculation simplifies to the cosine of one-half the phase angle.

When the phase angle is 180°, the cosine of one-half this
angle is the cosine of 90°, which is zero. The intensity of the resultant will then be zero and illumination will be at a minimum.

When the illumination from a point source in the condenser is split and recombined in the Baker interference microscope, the amplitude is unchanged and is therefore the same for the resulting pair of interfering waves. As shown previously, by rotating the analyser, the phase relationship between the pair of waves can be varied.

If the analyser is rotated until the observed object shows minimum illumination in monochromatic light, the phase difference of wave pairs from the object is 180° and the plane of polarisation of the analyser is, in effect, at right angles to the resultant of the two waves. The location of this resultant is midway between the two interfering waves. If the analyser is now rotated until the illumination of the background is at a minimum, the plane of polarisation of the analyser is at right angles to the resultant of the background waves.

The difference between two settings of the analyser required to render the object and the background black, in turn, is equal to one-half the phase difference caused by the refractive index difference between the object and the surrounding medium (2).

Substitution in the suitable formula, using twice the measured phase changes, for each of two media, gives the refractive index of the object (2).
The formula is as follows:
\[ u = A \left( \frac{n_2 - n_1}{A - B} \right) + n_1 \]

where 
- \( u \) is refractive index of the object 
- \( n_1 \) is the refractive index of the first medium 
- \( n_2 \) is the refractive index of the second medium 
- \( A \) is the phase change measured with the first medium 
- \( B \) is the phase change measured with the second medium.

CHANGES IN REFRACTIVE INDEX

Wood is a complex organic substance consisting of cells of various types. The basic structure of these cells is composed of cellulose and the cells are stiffened and held together by an encrusting matrix of lignin. In addition, various other materials, grouped under the name of hemicelluloses, are distributed through the cell structure. The essential process in pulping consists of dissolving out the lignin, freeing the cellulose cells from each other. In the process, some of the hemicellulose is also lost. If the pulp is bleached, further changes take place in the residual cellulose. There is some degradation due to rupture of the long-chain molecules of which the cellulose is composed. More of the hemicellulose is removed if alkaline solutions are used. The process of pulping and bleaching, then, causes drastic changes in the molecular structure.

As noted previously, refractive index is considered to be a function of molecular structure. An appreciable change in this structure should occur in wood that was converted to bleached pulp. If the
associated change in refractive index did occur, and could be measured and correlated with the degree of chemical treatment given to known samples of wood pulp, much might be learned about the process of chemical breakdown. In any event, if refractive index does change with change in molecular structure, it should be evident in the process of pulping and bleaching wood.

The refractive index of wood pulp can be measured, in theory, by using a Becke line test. However, this would require the transfer of individual tracheids from one to another media of different refractive indices over a definite range, bracketing the index of the pulp. Many of these would undoubtedly have undesirable characteristics such as low volatility or high viscosity. Changing media by evaporation would be difficult, as would the mechanical transfer of a single tracheid from one medium to another. The Baker interference microscope offered a solution to these problems.

EXPERIMENTAL MATERIAL

The pulp used consisted of western hemlock (Tsuga heterophylla (Rafn.)Sarg.) viscose pulp, removed at the end of each of several operations during the run of the same batch through the pilot plant of the laboratory at the Columbia Cellulose Co., Ltd. at Prince Rupert.

Samples of the pulp were collected after each individual operation of washed raw pulp, chlorination, refining and hot alkali extraction, cold caustic extraction and final bleaching. The viscosity and brightness of the various samples, as measured at the laboratory where prepared, are shown in appendix Table 5. Details of the various
stages of treatment were not released by the laboratory.

DETERMINATION OF REFRACTIVE INDICES

The pulps were received in the wash water in which they were removed from the pilot plant. Refractive indices of the wash water were determined on an Abbe refractometer. The results indicated a high degree of purity so the pulp was not washed further.

In order to measure refractive index by the method of immersion, it was necessary to first dry the pulp, since water, which evaporates slowly, was not a suitable medium for the first immersion. The pulp was dried for fourteen hours at 60°C. The first objective was to determine the practicability of the system of measurement being tried. If this method proved successful, it would then prove necessary to examine the effects on the pulp of flash drying, which would normally be used for the sake of speed.

Using the Becke line test and immersing a separate dried tracheid in each medium, the refractive index was determined for each sample. The same tracheid was not used in each medium so the comparability of the results is open to some question. However, since in all cases the refractive index fell between 1.55 and 1.56, it was felt these would serve as control limits for the measurements with the interference microscope.

Using the interference microscope, readings were taken for ten fibres from each of the dried samples. Either acetone or ethyl alcohol was used for the first, or volatile medium, and a liquid of high refractive index for the second.
### Table 1

Summary of Refractive Index Measurements by the Baker Interference Microscope

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Pulp No. 1</th>
<th>Pulp No. 2</th>
<th>Pulp No. 3</th>
<th>Pulp No. 4</th>
<th>Pulp No. 5</th>
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<tbody>
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<td>1</td>
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<td>1.49</td>
<td>1.44</td>
<td>1.57</td>
<td>1.63</td>
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</tr>
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<td>1.45</td>
<td>1.57</td>
<td>1.51</td>
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</tr>
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</tr>
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<td>1.45</td>
<td>1.65</td>
<td>1.56</td>
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<td>10</td>
<td>1.44</td>
<td>1.47</td>
<td>1.46</td>
<td>1.53</td>
<td>1.62</td>
</tr>
</tbody>
</table>

Pulp No. 1 - Raw Pulp  
Pulp No. 2 - After Chlorination  
Pulp No. 3 - After Refining and Hot Caustic Extraction  
Pulp No. 4 - After Cold Caustic Extraction  
Pulp No. 5 - After Secondary Bleaching
Refractive index readings were taken, using an Abbe refractometer, for the acetone and ethyl alcohol used and also for the medium of refractive index 1.63. The medium of index 1.77 was beyond the range of the instrument.

Readings on the pulp are summarized in text Table 1 (page 40) and detailed in appendix Table 3. Index readings on the media are shown in appendix Table 4.

RESULTS AND EVALUATION

The refractive index values, obtained with the interference microscope, varied considerably from the values obtained with the Becke line test. With very few exceptions, they exceeded the limits of 1.55 and 1.56. The results were considered to be outside of the limits of the true values. Measurements were taken to sufficient significant figures to permit of calculation of refractive index values to at least three decimal places. Because of the unsatisfactory values obtained, such refinement in the figures presented was not considered to be justified here.

A possible explanation of the failure of the technique to give the expected results can be found in the work of Mease (14) and of Sheppard and Newsome (22) as reported in (31). They reported adsorption by cellulose, cotton and rayon of various aliphatic alcohols, including methyl. The alcohols were not completely removed at elevated temperatures or high vacuum over extended periods. Also, Russell, Maass and Campbell (21) and Brimley (4), as reported in (18), report strong bonding of organic solvents with sulphite and kraft pulp and with cotton.

It is probable that the acetone or methyl alcohol used was
absorbed by the cellulose in the pulp and was not all evaporated. The medium of refractive index 1.63 consisted of alpha-monomobromonaphthalene diluted with clove oil. The medium of refractive index 1.77 was sulphur saturated methyl iodide. While the acetone or alcohol probably did not react with either of these chemical compounds, the possibility cannot be ruled out. In any case, the absorbed acetone or alcohol would act as a diluent, changing the refractive index of the second medium by an unknown amount.

A further complication is the differential swelling of cellulose in various liquids. Nayer (17) studied swelling in whole wood of eastern white pine (Pinus strobus L.) and of Sitka spruce (Picea sitchensis (Bong.) Carr.). Using the swelling in water as a base, arbitrarily set at 100 per cent, he found that the amount of swelling ranged from 0 per cent for benzene to well over 150 per cent for various other organic liquids. The value for acetone was 63 per cent and for ethyl alcohol was 83 per cent. The formula used assumes a constant thickness for the fiber. If this thickness varied for immersion in the two different media, it would cause inaccurate results.

The refractive index values obtained were analysed statistically as detailed in appendix Table 1 and summarised below. The analysis yielded a value of 0.34 as the largest difference that could be accepted without significant difference at the 5 per cent level between adjacent totals of samples when arranged in rank according to size. For individual, non-ranked comparisons this value was 0.80.
The ranked totals were as follows:

Pulp 3 14.57
Pulp 1 14.71
Pulp 2 14.85
Pulp 4 15.66
Pulp 5 16.00

Significance is reached between pulps 3 and 4 in ranked comparisons and between pulps 5 or 4 on one hand and pulps 1, 2 or 3 on the other in unranked comparisons. These results, in themselves, would indicate a change in refractive index due to treatment but no conclusions can be drawn because the refractive index values obtained differed from the control limits set by the Becke line test.

In the interference microscope used, the point of minimum illumination, on which phase measurements were based, was determined visually as a relative value. Although each reading was checked carefully by repetition and by approaching the minimum value from both directions by reversing the direction of travel of the analyser, the system is still subject to excessive human error. Subsequent to the completion of the experimental measurements and the return of the instrument to the owner, the manufacturer announced the availability of a half-shade eyepiece which would permit of the determination of the position of minimum illumination by a comparison between two halves of a split field. This should greatly increase the accuracy of the instrument.
V CONCLUSIONS

The interference microscope constitutes an important contribution to the science of microscopy since it eliminates many of the defects found in other systems. It provides visual images of bleached, unstained pulp superior to all the other methods tried.

No definite conclusion could be reached in the attempt to evaluate the application of the phase measurement system to the determination of refractive index in wood pulp. However, it is felt that the results were sufficiently good to warrant a continuation of the study when an interference microscope again becomes available. Probably the major difficulty to be overcome is the finding of media without objectionable properties.
LITERATURE CITED


Table 1

Statistical Analysis of Differences in Refractive Indices Between Pulps

<table>
<thead>
<tr>
<th>Term</th>
<th>Degrees of Freedom</th>
<th>Sum. Squares</th>
<th>Mean. Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>4</td>
<td>0.1602</td>
<td>0.0400</td>
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<tr>
<td>Error</td>
<td>45</td>
<td>0.0674</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

Least Significant Difference: for unranked comparisons = 0.81
(Between totals) for ranked comparisons = 0.35
Level of Confidence: 95%

Totals as found

<table>
<thead>
<tr>
<th>Pulp No.</th>
<th>Value</th>
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<tbody>
<tr>
<td>No. 1</td>
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</tr>
<tr>
<td>No. 2</td>
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<tr>
<td>No. 4</td>
<td>15.66</td>
</tr>
<tr>
<td>No. 5</td>
<td>16.00</td>
</tr>
</tbody>
</table>

Differences as found

| Pulp 5 minus Pulp 4 | = 0.34 |
| Pulp 4 minus Pulp 3 | = 1.09* |
| Pulp 3 minus Pulp 2 | = -0.23 |
| Pulp 2 minus Pulp 1 | = 0.14 |
| Pulp 5 minus Pulp 1 | = 1.29* |
| Pulp 5 minus Pulp 2 | = 1.15* |
| Pulp 5 minus Pulp 3 | = 1.43* |
| Pulp 4 minus Pulp 1 | = 1.09* |
| Pulp 4 minus Pulp 2 | = 0.95* |
| Pulp 4 minus Pulp 3 | = 0.81* |

* indicates significance at the 95% level of confidence.
Table 2

Refractive Index Measurement by Becke Line Test

Note: G indicates the refractive index of the pulp exceeded that of the immersion medium.

L indicates the refractive index of the pulp was less than that of the immersion medium.

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Index Medium</th>
<th>Pulp No. 1</th>
<th>Pulp No. 2</th>
<th>Pulp No. 3</th>
<th>Pulp No. 4</th>
<th>Pulp No. 5</th>
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<td>L</td>
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<td>2</td>
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Table 3

Phase Change Measurements with the Interference Microscope

Pulp at Stage No. 1 - Raw Pulp

<table>
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<tr>
<th>Trial No.</th>
<th>Immersion Medium</th>
<th>Goniometer Reading</th>
<th>Condition of Minimum Difference</th>
<th>Optical Path Illumination</th>
<th>Refractive Index</th>
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</table>

1. MeOH is Methyl alcohol.
Table 3 (Continued)

Phase Change Measurements with the Interference Microscope

Pulp at Stage No. 2 - after Chlorination

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Immersion Medium</th>
<th>Goniometer Reading</th>
<th>Optical Path Difference</th>
<th>Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Illumination</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Background</td>
<td>Fiber</td>
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<td>226</td>
<td>129</td>
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<td>18</td>
<td>111</td>
<td>96</td>
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<td>86</td>
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<td>125</td>
<td>208</td>
<td>83</td>
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<td>18</td>
<td>-110</td>
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Table 3 (Continued)

Phase Change Measurements with the Interference Microscope

Pulp at Stage No. 3 - after Refining and Hot Alkali Extraction

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<tr>
<th>Trial No.</th>
<th>Immersion Medium</th>
<th>Goniometer Reading</th>
<th>Optical Path Difference</th>
<th>Refractive Index</th>
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<td></td>
</tr>
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<td></td>
<td>Background Fiber</td>
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<td>142</td>
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<td>227</td>
<td>-163</td>
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<td>213</td>
<td>98</td>
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<td>Acetone</td>
<td>131</td>
<td>225</td>
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<td>51</td>
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Table 3 (Continued)

Phase Change Measurements with the Interference Microscope

Pulp at Stage No. 4 after Cold Caustic Extraction

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<th>Trial No.</th>
<th>Immersion Medium</th>
<th>Goniometer Reading</th>
<th>Condition of Minimum Illumination</th>
<th>Optical Path Difference</th>
<th>Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Background Fiber</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>Acetone 1.77</td>
<td>228 180</td>
<td>301 107</td>
<td>73 -73</td>
<td>1.57</td>
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<td>2</td>
<td>Acetone 1.77</td>
<td>212 169</td>
<td>310 36</td>
<td>98 -133</td>
<td>1.53</td>
</tr>
<tr>
<td>3</td>
<td>Acetone 1.77</td>
<td>126 221</td>
<td>164 179</td>
<td>38 -42</td>
<td>1.55</td>
</tr>
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<td>4</td>
<td>Acetone 1.77</td>
<td>127 213</td>
<td>175 165</td>
<td>48 -48</td>
<td>1.57</td>
</tr>
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<td>Acetone 1.77</td>
<td>174 212</td>
<td>208 175</td>
<td>34 -37</td>
<td>1.56</td>
</tr>
<tr>
<td>6</td>
<td>Acetone 1.77</td>
<td>120 182</td>
<td>189 102</td>
<td>69 -80</td>
<td>1.55</td>
</tr>
<tr>
<td>7</td>
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<td>217 110</td>
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</tr>
<tr>
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<td>56 88</td>
<td>49 -69</td>
<td>1.65</td>
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<tr>
<td>9</td>
<td>Acetone 1.77</td>
<td>99 153</td>
<td>114 97</td>
<td>50 -56</td>
<td>1.55</td>
</tr>
<tr>
<td>10</td>
<td>Acetone 1.77</td>
<td>109 234</td>
<td>171 146</td>
<td>62 -88</td>
<td>1.53</td>
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</table>
Table 3 (Continued)

Phase Change Measurements with the Interference Microscope

Pulp at Stage No. 5 after Secondary Bleaching

<table>
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<th>Trial No.</th>
<th>Immersion Medium</th>
<th>Goniometer Reading</th>
<th>Optical Path Difference</th>
<th>Refractive Index</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Background Fiber</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Acetone 1.77</td>
<td>182 311</td>
<td>129</td>
<td>1.63</td>
</tr>
<tr>
<td>2</td>
<td>Acetone 1.77</td>
<td>145 145</td>
<td>100</td>
<td>1.58</td>
</tr>
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<td>3</td>
<td>Acetone 1.77</td>
<td>125 186</td>
<td>61</td>
<td>1.71</td>
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<td>Acetone 1.77</td>
<td>144 170</td>
<td>26</td>
<td>1.51</td>
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<td>5</td>
<td>Acetone 1.77</td>
<td>195 213</td>
<td>18</td>
<td>1.55</td>
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<td>175 280</td>
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<td>1.61</td>
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<td>Acetone 1.77</td>
<td>137 197</td>
<td>60</td>
<td>1.56</td>
</tr>
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<td>9</td>
<td>Acetone 1.77</td>
<td>167 99</td>
<td>68</td>
<td>1.55</td>
</tr>
<tr>
<td>10</td>
<td>Acetone 1.77</td>
<td>147 51</td>
<td>96</td>
<td>1.62</td>
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</table>
### Table 4
Refractive Indices as Measured by the Abbe Refractometer

<table>
<thead>
<tr>
<th>Wash Water/ Medium of Index</th>
<th>Pulp No. 1</th>
<th>Pulp No. 2</th>
<th>Pulp No. 3</th>
<th>Pulp No. 4</th>
<th>Pulp No. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Water:</td>
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<td>1.3321</td>
<td>1.3320</td>
<td>1.3320</td>
<td>1.3320</td>
</tr>
<tr>
<td>Medium of Index 1.63</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl Alcohol</td>
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</tr>
</tbody>
</table>

### Table 5
Brightness and Viscosity Measurements of Pulps
as Taken at Laboratory where Prepared

<table>
<thead>
<tr>
<th>Pulp No.</th>
<th>Viscosity</th>
<th>Brightness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulp No. 1</td>
<td>149</td>
<td>55.2</td>
</tr>
<tr>
<td>Pulp No. 2</td>
<td>149</td>
<td>57.0</td>
</tr>
<tr>
<td>Pulp No. 3</td>
<td>200</td>
<td>71.6</td>
</tr>
<tr>
<td>Pulp No. 4</td>
<td>197</td>
<td>77.5</td>
</tr>
<tr>
<td>Pulp No. 5</td>
<td>58</td>
<td>92.2</td>
</tr>
</tbody>
</table>