THE TECHNIQUE OF ULTRA-VIOLET ABSORPTION SPECTROSCOPY WITH THE BURCH REFLECTING MICROSCOPE

by

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INTRODUCTION

The construction of a high performance reflecting microscope by BURCH (BURCH 1947, BARER 1948) is an important landmark in the development of microscopy. The numerous applications of this, and other types of reflecting microscope, will not be discussed here. However, in view of the growing interest in reflecting objectives and the likelihood that more microscopes of the BURCH type will be available in the future, we have felt it desirable to record in some detail our experiences with a new and simple method of absorption spectroscopy of microscopical objects (spectromicrography), which has been made possible by the perfect achromatism of the BURCH microscope. While some of the methods described may be fairly familiar to professional spectroscopists, they will be less so to most biologists. This paper will be confined to certain practical problems concerned with the new technique. The detailed results of investigations on various types of material will be discussed elsewhere.

THE BURCH REFLECTING MICROSCOPE

This instrument differs from other reflecting microscopes hitherto described in that it utilizes accurately worked aspherical surfaces. The instrument in use at Oxford is shown diagrammatically in Fig. 1. The objective is composed of a small convex, nominally spherical, mirror m_o and a large concave aspherical mirror M_o. Its numerical aperture (N.A.) is 0.65. The condenser is similar, but in the present instrument its N.A. is 0.58. The N.A. of the objective can be increased to 0.98 by means of a normal-incidence immersion lens component. This does not affect the achromatism of focus but introduces a small amount of chromatic error of magnification. The latter is of no consequence in the technique described below, since it is in any case present in the spectrograph. However, in the present investigation the instrument was used without the immersion component. The use of aspherical surfaces results in two important advantages: – (i) it makes possible the construction of objectives of high N.A., with

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a minimum of spherical aberration, (ii) the obstruction ratio, *i.e.*, the proportion of the aperture of the objective which is lost because of the shadowing effect of the small mirror, can be kept low. These points require further explanation.

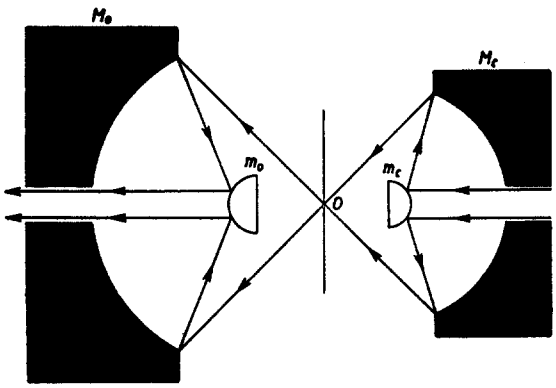

Fig. 1. Diagram of BURCH reflecting microscope in its simplest form. *Mo*, *mo* objective mirrors; *Mc*, *mc* condenser mirrors; *Mo* and *Mc* are aspherical

It is relatively simple to construct a reflecting objective composed of two spherical mirrors, but spherical aberration becomes excessive at N.A. greater than 0.5. The use of aspherical mirrors enables the N.A. to be increased (in principle at least) to values approaching 1.0. These figures can be increased by a factor of n : 1, when an immersion component is used (n is the refractive index of the lens component). With a spherical mirror objective of N.A. 0.5 (BURCH 1947) the obstruction ratio is generally about 40%. The effect of this obstruction is to alter the energy distribution in the Airy disc pattern. The central maximum is decreased in intensity and there is a relative increase in the lateral maxima. The practical result of this is not always easy to predict, since it depends on the type of object, but with biological objects such as cells there is generally a loss of contrast which may lead to reduction of resolution. The effect becomes rapidly more serious for obstruction ratios exceeding 20% (RAYLEIGH 1902, BOUWERS 1946, HOPKINS 1949). The advantages of aspherical surfaces which allow the construction of objectives of higher aperture and at the same time of low obstruction ratio will thus be apparent. The obstruction ratio of the microscope actually used is 14%.

The objective mirrors are mounted independently, with screw controls which enable the system to be corrected for coma by moving the mirrors relative to one another in a plane perpendicular to the principal axis, and for spherical aberration by movements along this axis. The latter adjustment is extremely important since it enables the objective to be corrected for different thicknesses of cover-slip, and for use at different projection distances. The mirrors are constructed of speculum metal, but as this is a poor reflector of ultraviolet light, a very thin surface layer of aluminium is deposited on each mirror *in vacuo*.

SPECTROMICROGRAPHY (MICROSPECTROGRAPHY)

Spectroscopic methods in microscopy have been employed for many years. Spectroscopic eyepieces incorporating Amici direct-vision prisms were constructed by BROWNING AND SORBY and by ABBE, more than fifty years ago. Such instruments have been extensively used for work on biological pigments and coloured specimens, but their use is restricted to the visible region of the spectrum. A considerable advance in technique was made by JELLEY (1936) who developed an eyepiece spectroscope with a diffraction grating in place of the Amici prism. This instrument incorporated an ingenious method of photographic recording and was used for studies on birefringence and dichroism as well as conventional spectroscopy. Another method of spectromicrography, rarely used,

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employs a spectroscopic condenser which projects an image of the spectrum on to the field of view.

A somewhat different approach has been used by CASPERSSON and others, in the ultraviolet region (CASPERSSON, 1936, 1940, THORELL, 1947, COLE AND BRACKETT 1940). In principle the method depends on taking a series of photographs of the same object in monochromatic ultraviolet light of different wavelengths. The absorption of light at each wavelength is then measured by means of a microphotometer. A modification of this method is to measure the transmission directly by photoelectric means, without the use of photography (CASPERSSON, 1947, POLLISTER AND RIS 1947, GERSH AND BAKER, 1943). This type of technique has been made necessary because until recently quartz-lens microscopes have had to be used for ultraviolet microscopy. Such instruments are corrected for use at one wavelength only (usually 2750 A.U.) but for spectroscopic work it is desirable to cover a wide range of wavelengths. Some workers (POLLISTER AND RIS, 1947) have used the absorption at one single wavelength as a measure of the concentration of certain substances, but this is clearly undesirable and may easily lead to errors. For work on nucleic acids and proteins in cells a minimum range extending from 2500 A.U. to 3000 A.U. is required. In order to cover this range with the quartz microscope a number of monochromatic exposures have to be made at various wavelengths. This introduces numerous difficulties. In the first place, in order to obtain a reasonably smooth absorption curve the wavelengths used should be close together and fairly evenly spaced. The intensity of the illumination after passage through the monochromator must be sufficient to allow accurate focussing and a reasonably short exposure. These requirements are difficult to fulfil with a single light source, and it is sometimes necessary to use two or more different sources (mercury arc, cadmium spark etc.) in order to cover the spectral range required. Since the quartz lenses are corrected for use at one wavelength only, the performance of the microscope inevitably deteriorates when it is used at other wavelengths. This deterioration, due to spherical aberration, becomes more marked the further away the objective is used from the correct wavelength.

SPECTROMICROGRAPHY WITH THE REFLECTING MICROSCOPE

A unique feature of the reflecting microscope is its true achromatism, all wavelengths reflected by the mirrors forming images in the same plane. With such an instrument it becomes possible to disperse the radiation *after* it has passed through the object, instead of before, thus obtaining a record of spectral absorption of the latter simultaneously at all wavelengths emitted by the source. We have achieved this by projecting the image from the microscope onto the entrance slit of a spectrograph; the spectrograph forms a series of images of the slit (and that portion of the microscope image which lies across it) dispersed according to wavelength. Each point across the slit is represented in the spectrogram by a series of points which form a spectrum. In this way we are able to determine the spectral absorption of any elementary area of the object. The factors which govern the size of the smallest area from which an accurate absorption record can be obtained have been discussed by CASPERSSON (1936), who concludes that this minimal area has a diameter about four times the wavelength.

It might be argued that since in this procedure the entire radiation from the source is passing through the specimen the latter is being unduly exposed to any destructive action of ultraviolet light. This argument loses its force when it is remembered that in

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order to obtain an absorption spectrum the object must in any case be exposed to radiation at a number of wavelengths. By dispersing the light *after* passage through the object it is possible to obtain a record *simultaneously* at many wavelengths. It thus becomes possible in principle to make observations on material in motion. If separate observations are made at each desired wavelength, as is necessary when using a non-achromatic microscope, the state or position of the object may have changed during the interval between the early and later observations. The exposure of the object to ultraviolet light for the purpose of focusing trials is of course eliminated by the use of a reflecting microscope.

A modification of this method has been applied to the infra-red region by BARER, COLE AND THOMPSON (1949). In principle it should be possible to work with radiation of any wavelength which is reflected by the mirrors, and for which sufficiently sensitive methods of detection are available. It must be remembered however that the resolving power of the microscope, and the size of the smallest area on which accurate absorption measurements can be made are both adversely affected by increase in wavelength. The range of wavelengths so far covered extends from below 240 mμ in the ultraviolet to 14 μ in the infra-red.

EXPERIMENTAL

At first experiments were carried out in the visible region using a standard microscope with achromatic and apochromatic lenses. The method was later extended to the ultraviolet region using the reflecting microscope. Preliminary examples of the records obtained (including spectrograms) have been given elsewhere (JOPE 1949, BARER 1949).

Adjustment of microscope

The reflecting microscope is set up with its axis vertical, so that wet preparations may be used without risk of movement of the object due to gravity. An image of the source is formed by means of a quartz lens on the condenser aperture stop (KÖHLER illumination). A field stop is used in order to limit the size of the field illuminated and to improve contrast. Before beginning a series of observations it is advisable to check the mirror adjustments by examining a test slide. The minute pinholes in a silver film deposited on a glass slide (with or without coverslip) are very convenient objects for studying the Airy disc pattern, and for detecting the presence of coma and spherical aberration. These aberrations are minimised by adjustment of the relative positions of the objective mirrors, until the Airy disc pattern is circular and as nearly as possible symmetrical above and below focus (Star test). These adjustments must be carried out for the projection distance at which the microscope is being used, and must be repeated whenever this distance is altered. The test slide is now replaced by the specimen slide and an image of the specimen is projected by means of a surface aluminised mirror onto the entrance slit of the spectrograph (Fig. 2a, b). The projection mirror has an adjustable angle of tilt, and can be rotated about a vertical axis.

Focusing

With a Mercury arc or tungsten filament lamp as source, the illumination is sufficiently intense to allow focusing and exact location of the image relative to the slit. The image is observed directly on the jaw plates of the slit, which should be painted white. With less intense sources, such as the hydrogen arc, focusing can be carried out by direct vision through an eyepiece, and this is sometimes desirable even with more intense sources. Ideally, the spectrograph should be modified to enable an eyepiece to be introduced behind the slit, but where this is not possible, the image may be swung to one side of the slit by means of the rotatable projection mirror, focused by means of the eyepiece and then swung back to cover the slit. The image may also be examined in its final setting as a spectrum, with an eyepiece in the focal plane of the spectrograph. This is often helpful in defining the exact portion of the specimen which lies across the slit. A very useful alternative procedure when weak sources are used is to set up the source in alignment with the microscope, but to carry out focusing and image location by means of light from a tungsten filament lamp, which is sent into the system by a 45° mirror which can be swung out of the way when not required (M' in Fig. 2b). Since many of the objects studied, particularly living cells, are transparent to visible light, some difficulty may be experienced in obtaining a sharp image. The ideal method for focusing such objects

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would be to form an image by phase-contrast illumination, making due allowance for any change of focus caused by the introduction of the phase plate. It is intended to employ this method in future work. In all methods of focusing, a glass plate or a coloured glass filter is placed in front of the source in order to protect the object from the effects of ultraviolet radiation. No alteration of focus is required when this plate or filter is removed for the exposure of the spectrogram.

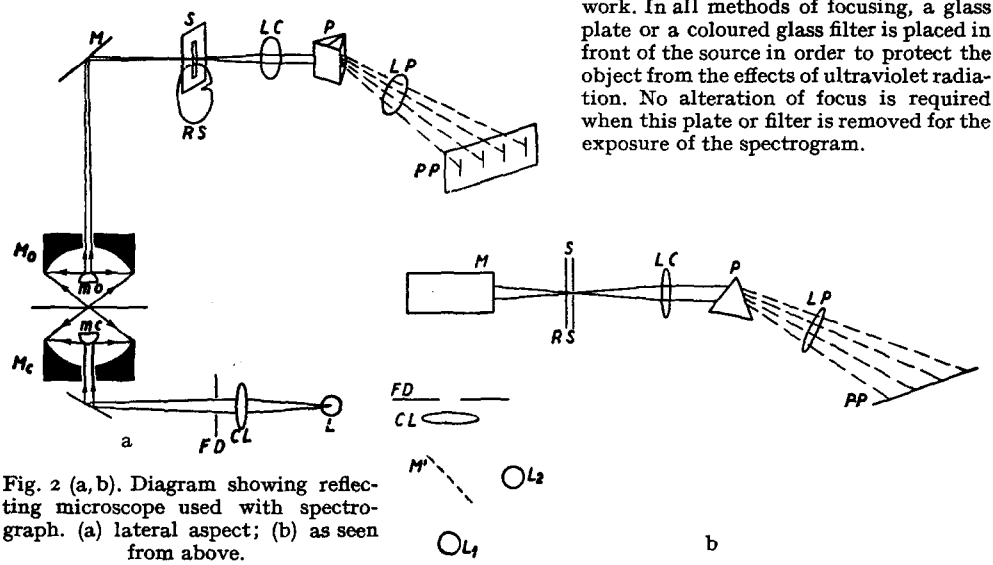

Fig. 2 (a, b). Diagram showing reflecting microscope used with spectrograph. (a) lateral aspect; (b) as seen from above.

L_1 main source
L_2 auxiliary source for focusing
CL condensing lens
FD field diaphragm
M projection mirror
RS Rotating logarithmic sector
S Spectrograph slit
LC collimating lens
P prism
LP camera lens of spectrograph
PP photographic plate

Adjustment of the spectrograph

This procedure calls for little special comment. It is usually best to open the slit rather widely, and to pick up the image of the source visually by observing the aperture of the camera lens directly, and to move the spectrograph in the vertical and horizontal planes until the image is centrally placed in this aperture. The spectrum itself can then be observed by means of an eyepiece focused on the focal plane of the spectrograph. The slit is then adjusted to the desired length and width. It is perhaps necessary to point out that the type of record obtained from the spectrograph depends on the way it is used. Thus the most usual method is to employ the spectrograph as a spectral analyser. For this purpose a source giving a continuous spectrum is preferable. The slit of the spectrograph is made narrow (0.02–0.1 mm) and the final spectrogram becomes that of the emission spectrum of the source, superimposed upon which is the absorption spectrum of that part of

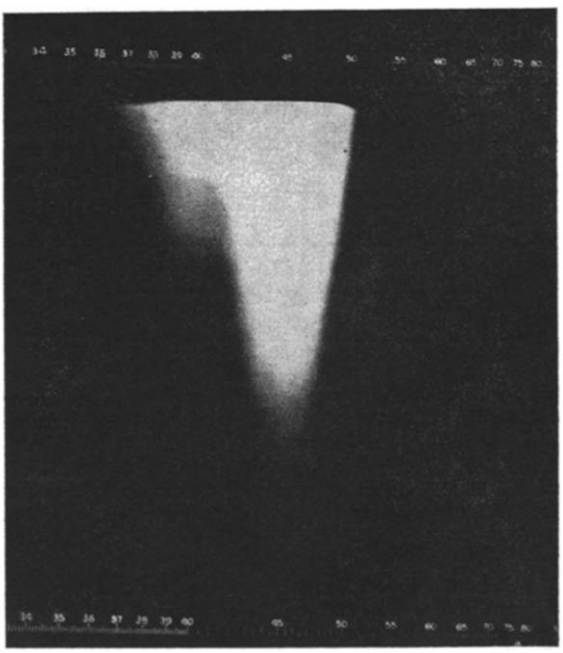

Fig. 3. "Moving plate" spectrogram of a small central area of the microscope image of a single human red blood cell, showing the Soret absorption band of Oxyhaemoglobin, at 414.5 mμ (Tungsten lamp source)

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the object which is imaged across the slit. This use of the spectrograph has been extended. The slit can be shortened until its length becomes of the same order as its width, so that a very small portion of the microscope image is isolated. We have applied the moving plate method (HOLIDAY 1937) for the qualitative analysis of portions of a cell as small as is allowable by the limits of resolution of the microscope (Fig. 3). This method, in which the photographic plate is moved across the spectral image at a logarithmically increasing rate, gives in effect a silhouette of the absorption spectrum of the object. It is particularly valuable for detecting spectral fine structure.

During the course of this work it became clear that the spectrograph could in some cases be used in a rather different way, namely as a *prismatic camera*. This is only possible with sources of radiation showing a few strong lines well displaced from one another, *e.g.* the Mercury arc. The slit is made wide (of the order of several millimetres) and the final record is in the form of a set of simultaneous monochromatic photographs of extended areas of the object. This technique is in some ways reminiscent of that used by astronomers in the spectroheliograph (see DIMITROFF AND BAKER, 1945). It may be possible to develop this method still further by moving the object or the spectrograph in such a way as to obtain simultaneous monochromatic pictures of very large areas of the object. It should be pointed out of course, that the record obtained with a narrow slit and continuous spectrum can be regarded as an *infinite* number of simultaneous monochromatic photographs of narrow regions of the object. This interpretation serves to emphasize the peculiar advantage of dispersing the radiation after passage through the object. If this were not done the same information could only be obtained photographically by taking an infinite number of separate exposures.

Density Calibration with Rotating Sector

For qualitative and semi-quantitative work it is often sufficient to compare the relative absorptions of different parts of the same object at various wavelengths. Where accurate measurements of spectral absorption are required however, it is necessary to have for comparison a record of the response of the photographic plate to exposures varying in a known way. For this purpose we have found the rotating sector method very convenient. The sector we have used up to the present is a standard logarithmic pattern (Hilger), giving an optical density range of 0.3 to 2.3 corresponding to transmission by 50% of 360° at its circumference and 0.5% of 360° at the innermost point, 1.3 cm from the circumference. This is not quite ideal for the density range encountered when examining the ultraviolet spectral absorption of objects such as biological cells, and a sector giving a range of optical density of 0.1 to 1.3, over a length of, say, 0.7 cm (corresponding to 80% of 360° transmission at the circumference and 5% of 360° at 0.7 cm from the circumference) would be more suitable.

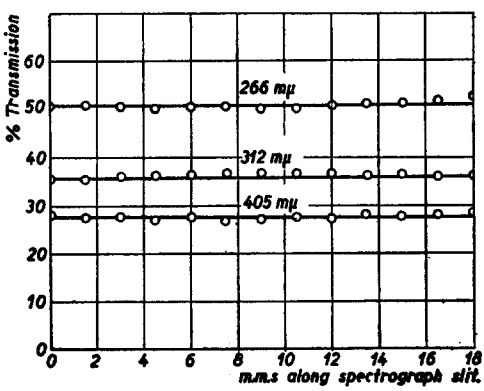

Fig. 4. Densitometer plot across slit image on a test spectrogram, showing evenness of illumination

The sector is set up immediately in front of the spectrograph slit, and covering the lower part of it. The image of the object is projected on the upper part of the slit, above the sector. It is a requirement of such a system that the illumination over the part of the field covering the length of the slit shall be uniform, and it is necessary to take a preliminary exposure without the object in position, in order to be certain that this condition is fulfilled. Fig. 4 shows a densitometer plot across such a test plate, at 3 wavelengths. This preliminary check for uniformity of illumination could of course be carried out more rapidly with a direct photoelectric scanning device. When a spectrogram of an object in the microscope is being exposed care must be taken to ensure that the part of the field in front of which the sector rotates is uniformly illuminated and is free from images of light-absorbing or scattering material. The use of a rotating logarithmic sector in the manner described, enables the optical density of any area in the image to be matched directly at any wavelength with a known optical density, recorded at the same wavelength, and in reasonable proximity on the photographic plate (BRODE 1943, SCHEIBE AND NEUHAUSER 1928, SCHEIBE 1925, 1929, TWYMAN AND SIMEON 1930).

Photographic process

It is not proposed to discuss here the relative merits of photographic and photoelectric methods of recording (see DIEKE 1947). For the present work photographic methods had certain definite advantages, and it was decided to use them, although photoelectric methods specially adapted for use with the reflecting microscope are in course of development. THORELL (1947) has shown that photographic methods can be made quite adequate for this type of work. We have tried out a large

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variety of photographic plates. In general the high contrast obtainable, especially below 270 mμ, with process plates such as the Kodak B10 and B20 series is very desirable. The Kodak 10 and 00 special emulsions are very useful where great sensitivity is required.

Illumination in the Ultraviolet

Where a source giving a line spectrum is used the relative intensities of the various lines may be widely different. This effect is quite marked with the high-pressure mercury lamp. Thus the lines at 266 and 366 mμ are very intense whereas the group of lines in the 280 mμ region is much less so. It may thus happen that on the same spectrogram one line is greatly overexposed while another is under-exposed. It may therefore be necessary to take two or more exposures of different lengths in order to obtain a good picture at each wavelength. In practice it is rarely necessary to take more than three such exposures, and where only a relatively narrow range of wavelengths is required, one exposure may be sufficient. With the continuous spectrum from a hydrogen lamp, the energy is fairly uniformly distributed and fewer exposures are required.

Whatever type of source is used, it is necessary to take into account two factors: – (1) the wavelength response of the photographic plate, (2) the variation in reflectivity of the mirror surfaces with wavelength. The available data suggest that the reflectivity of aluminium drops slowly but steadily as the wavelength becomes shorter. This effect may become quite important when the number of reflecting surfaces is large. In the present work six aluminized surfaces are employed. Two of these (the substage mirror and the projection mirror) might be dispensed with but there would always be a minimum of four surfaces. If we assume that the reflectivity of aluminium at 240 mμ is 90% of that at 400 mμ, the relative intensity of illumination at 240 mμ after passage through the microscope would be 0.9^6 or only 53% of that at the higher wavelength. This is not serious in practice as the effect of the fall-off at shorter wavelength can easily be overcome by giving a longer exposure. Ideally we should like a source giving a spectrum the intensity of which rises with shortening wavelength in such a way as to compensate for the fall in reflectivity. No such source exists in practice. There is no doubt that much work is required on the development of suitable sources for the full exploitation of the advantages of the reflecting microscope.

Although in principle the present technique should enable a complete absorption record to be obtained at all wavelengths between say 2200 AU and 1.3 μ by a *single* exposure, the various practical limitations discussed above make it necessary to cover so wide a range of wavelengths in a small number of exposures depending on the source, the photographic plate, and the absorption of the specimen.

Densitometry and analysis of records

A SIEGBAHN recording densitometer was used, similar to the one used in CASPERSSON's laboratory (THORELL, 1947). The scanning light spot was reduced to small dimensions, and adequate sensitivity assured by using a photomultiplier to actuate the recording galvanometer.

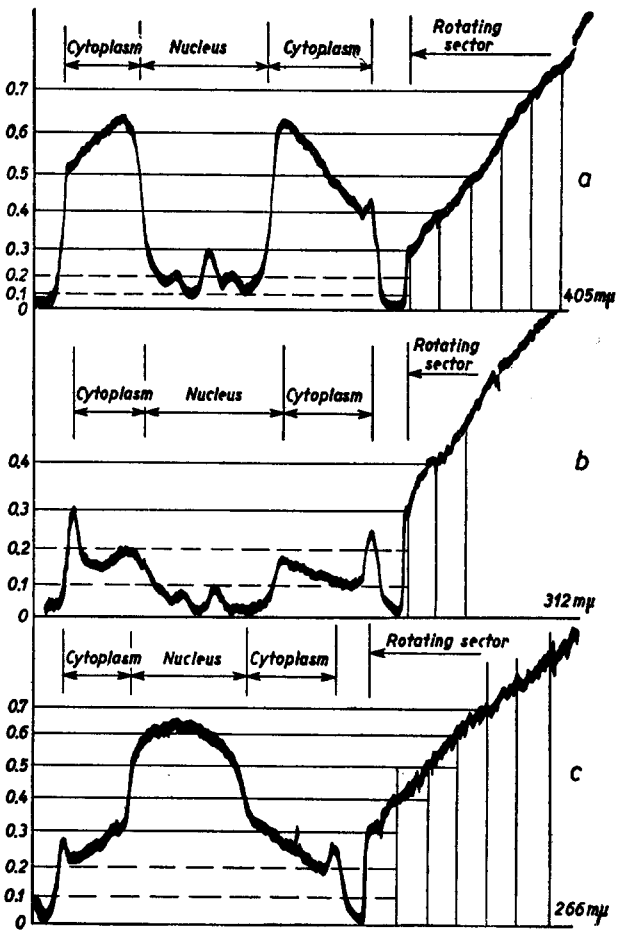

Fig. 5 (a, b, c). Recording densitometer plots across spectrogram image of Salamander red cell (nucleated) at three wavelengths. Note the heavy cytoplasmic absorption at 405 mμ close to the Soret band of haemoglobin. At 266 mμ on the other hand the nuclear absorption is greater. Density calibration from rotating sector on right of each figure (Medium pressure mercury lamp)

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Records may be obtained from the spectrograms in two ways.

1. If a line source is used scanning may be carried out down the length of each spectral line, the record across the object and logarithmic sector being used to derive the optical density of any given point in the object. This is done by dividing the whole length of the sector record (representing object densities 0.3 to 2.3) into equal divisions along the horizontal axis, corresponding to density increments, 0.3, 0.4, 0.5 etc. Verticals are then run up to the record trace, and parallels to the base line drawn at these intersects give the ordinates corresponding to these densities. Fig. 5 shows such an analysis of a record across the spectrogram of a Salamander red blood cell at three wavelengths, 405 mμ, 312 mμ and 266 mμ. From such records at a number of wavelengths, spectral absorption curves may be constructed for given points in the object: curves for the cytoplasm and nucleus of a Salamander red blood cell are shown in Fig. 6.

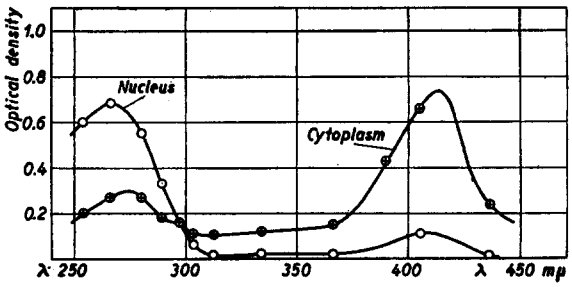

Fig. 6. Spectral absorption of points in the cytoplasm and nucleus of a Salamander red blood cell

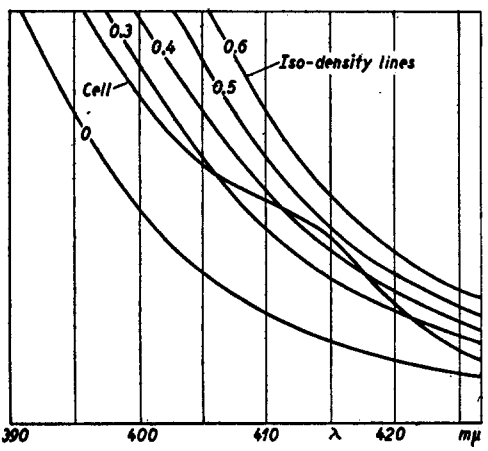

Fig. 7. Recording densitometer analysis of spectromicrogram of Salamander red blood cell cytoplasm using a continuous spectrum (tungsten filament lamp)

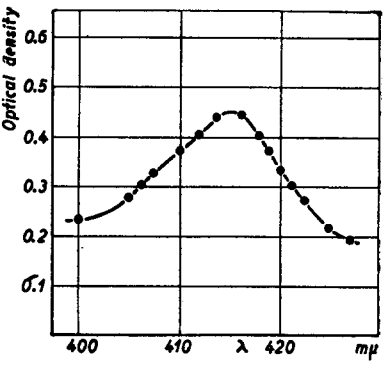

Fig. 8. Soret absorption band of a point in the cytoplasm of a Salamander red blood cell, constructed from Fig. 7

2. If a continuous source is used to obtain the spectrogram, the following procedure may profitably be used in addition to that described above. A number of records may be made all on the same sheet of photographic paper by scanning through the spectrum at a series of points in the object and sector images. A record along the spectrogram, of a point in the cytoplasm of a Salamander red blood cell, taken with a tungsten lamp is shown in Fig. 7 and from such a diagram the spectral absorption of a point in the cell may be constructed, as in Fig. 8, which was derived from Fig. 7. Such records take full advantage of the continuous spectral source, and in this way it is possible to obtain (a) the spectral absorption of any given point in the image, (b) the variation in absorption of all points in the image lying across the slit, at any wavelength. An analysis as complete as this can only be obtained readily by photographic methods of recording.

DISCUSSION

The most obvious advantage of the method described above is that it is possible to obtain an absorption record of the object *simultaneously* at a number of wavelengths. This has not been possible hitherto since truly achromatic objectives were not available.

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The new method results in a very considerable saving of time and effort. Quite apart from this, the ability to use a continuous spectrum is of fundamental importance, for only in this way is it feasible to obtain a smooth absorption curve without risk of uncertainty. This is an essential condition for any work on the "fine structure" of spectral absorption bands. While it is true that complicated absorption bands and small wavelength shifts are more frequently encountered in the infra-red region, nevertheless there are indications that their investigation may become of increasing importance in ultraviolet work. Thus it has been shown that whereas the spectral absorption of free tryptophan shows a prominent peak at 288 mμ, in most tryptophan-containing proteins the band is shifted to a longer wavelength, usually 291 mμ (HOLIDAY AND JOPE, 1949, JOPE 1949). Another remarkable example of the existence of small wavelength differences in the absorption spectra of biological materials has been observed in the case of red cell haemoglobins. In human foetal haemoglobin the tryptophan band occurs at 289.8 mμ whereas in foetal rat and sheep haemoglobin as well as in human adult material the maximum occurs at 291 mμ (JOPE 1949). Such small differences in wavelength could not possibly be detected and measured satisfactorily with anything but a continuous spectrum. Another field in which the investigation of spectral "fine structure" is likely to be of some importance is that of low temperature spectroscopy. It is well-known that the absorption bands of many substances show greater sharpness and complexity at very low temperatures (HOLIDAY, JOPE AND JOHNSON, unpublished observations, LOOFBOUROW, SCOTT AND SINSHEIMER 1948, BROWN AND RANDALL 1949). It may be possible to extend such observations to biological cells, and other materials.

Although in this communication we have been primarily concerned with the application of the technique to cytological material, it should be stressed that the method is equally applicable to the investigation of small quantities of material of any type. Thus it has been used on crystals, minute drops of liquid, fibres, films and waxy smears. An example of its application to microscopic crystals is shown in Fig. 9 in which the material used was a single crystal of 1, 2, 5, 6-dibenzanthracene. In this case, as well as in many others, differences have been observed between the spectra from the substance in the solid state and the same substance in solution. The method is particularly useful for work on solid substances in crystalline or powder form as difficulties due to scattering by the specimen are usually very much less serious than in the macroscopic technique. It has proved to be of value in the early stages of chemical isolation of new unknown compounds, when only very small quantities were available. An investigation of the ultraviolet, visible and infra-red absorption spectra may provide valuable structural information at such a stage, resulting in a saving of time and effort. The reflecting microscope enables

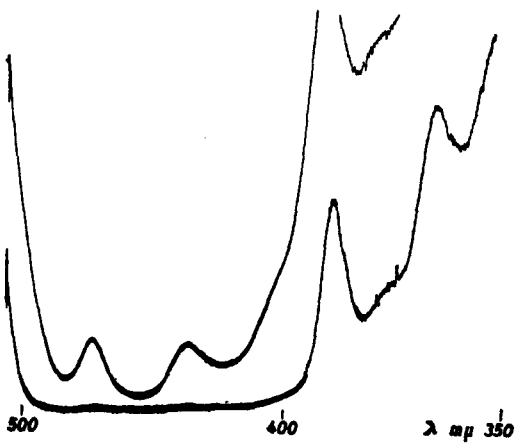

Fig. 9. Recording densitometer plots of spectromicrogram of minute crystal of 1,2,5,6-dibenzanthracene. The two lines are separate plots of two exposures of different length (Tungsten filament lamp).

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such an investigation to be carried out on the *same* specimen using the *same* microscope without disturbance.

It is perhaps necessary to indicate the order of sensitivity of spectromicrographic methods. The amount of haemoglobin in a single human red blood corpuscle has been calculated to be about $30 \cdot 10^{-12}$ g. It is quite easy to plot variations in distribution of haemoglobin in such a cell using the present method (Fig. 10). In general for ultraviolet work quantities as small as 10^{-12} g can be used, provided that the substance possesses a reasonably highextin ction coefficient.

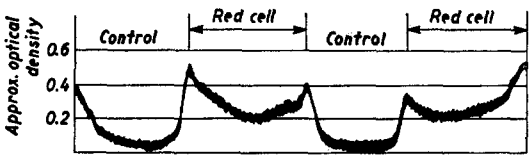

Fig. 10. Recording densitometer plot across spectromicrogram images of two human red blood cells, at the Soret band wavelength. Note that the absorption diminishes towards the centre of the cell. This is due to the biconcave shape of the latter (Hydrogen arc lamp).

Another way of looking at the problem is to consider the minimum amount of any given substance which can be used for absorption spectroscopy by conventional macroscopic methods. Let us assume that the sample is in the form of a cylinder of thickness t and radius r. The weight of the sample is then proportional to the volume $\pi r^2 t$. If the same sample is examined through the microscope its effective volume becomes $\pi (mr)^2 t$, where m is the linear magnification of the microscope. Thus the weight of the sample required for spectroscopy is reduced by a factor of m^2. If m is 400, the result is an increase in sensitivity of 160,000 times. The method is thus extremely delicate and enables results to be obtained on unweighable quantities of material in high local concentration. A noteworthy feature of the technique is its relative simplicity. Once the microscope, source, and spectrograph are set up, the spectral absorption of a minute sample can be recorded photographically with no more trouble than is required in taking an ordinary routine photomicrograph.

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SUMMARY

An account is given of various spectromicrographic techniques. The objective most commonly employed hitherto for ultraviolet microscopy have been quartz monochromats, which must be used with monochromatic light and are corrected for use at one wavelength only. These limitations introduce complications in the experimental technique and make it difficult to obtain smooth and accurate records of spectral absorption. These difficulties are a voided by the use of truly achromatic microscopes, such as the Burch reflecting microscope. In its simplest form this instrument contains no refracting elements and is achromatic over the entire spectral range. The use of an achromatic objective makes it possible to disperse the radiation *after* passage through the specimen. This is achieved by projecting the image of the object formed by the microscope onto the entrance slit of an appropri-

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ate spectrograph. By using a source such as a hydrogen discharge lamp, giving a continuous spectrum, it is possible to obtain a record of spectral absorption at every wavelength, and thus to study spectral fine structure.

Experimental details of the methods employed are given, together with indications of the types of results which can be obtained. The method has been used on a wide range of materials including cells, fibres and crystals, with both polarized and unpolarized light. The wavelength range so far covered extends from below 240 mμ to 14 μ.

RÉSUMÉ

Nous donnons un aperçu des diverses techniques spectromicrographiques. L'objectif généralement employé jusqu'ici en microscopie ultraviolette était constitué par des lentilles de quartz qui doivent être employées avec de la lumière monochromatique et corrigées pour l'emploi à une seule longueur d'onde. Ces limitations compliquent la technique expérimentale et rendent l'obtention de bonnes courbes difficile. L'on évite ces difficultés par l'emploi de microscopes vraiment achromatiques tels que le microscope à réflection de BURCH. Sous sa forme la plus simple cet instrument ne contient pas d'éléments réfracteurs et il est achromatique pour toutes les longueurs d'onde du spectre. L'emploi d'un objectif achromatique permet la dispersion des rayons *après* leur passage à travers le spécimen. On réalise ceci en projetant l'image de l'objet formée par le microscope sur la fente d'entrée d'un spectrographe approprié. En employant une source de lumière donnant un spectre continu, telle qu'une lampe à hydrogène, l'on peut enregistrer l'absorption spectrale à toutes les longueurs d'onde et étudier ainsi le detail de la structure d'un spectre.

Nous donnons des détails expérimentaux au sujet des méthodes employées ainsique des indications sur le genre de résultats auxquels on peut s'attendre. Nous avons appliqué la méthode décrite à des substances très diverses telles que des cellules, des fibres et des cristaux, avec de la lumière polarisée et non-polarisée. Le domaine de longueurs d'onde étudié jusqu'ici s'étend de 240 mμ à 14 μ.

ZUSAMMENFASSUNG

Die verschiedenen spektromikrographischen Arbeitsmethoden werden besprochen. Bisher wurden in der Ultraviolettmikroskopie meist Quartz-linsen verwendet; man arbeitet mit monochromatischem Licht und die Linsen sind auf *eine* Wellenlänge korrigiert. Hiedurch wird die Arbeitstechnik kompliziert und nur schwer erhält man gute und genaue Absorptionskurven. Diese Schwierigkeiten können durch den Gebrauch wahrhaft achromatischer Mikroskope, wie des Reflektionsmikroskopes von BURCH, vermieden werden. Dieses Instrument enthält in seiner einfachsten Form kein lichtbeugendes Element und ist für alle Wellenlängen des Spektrums achromatisch. Die Verwendung eines achromatischen Objektivs macht es möglich, das Licht zu streuen *nachdem* es die zu untersuchende Probe passiert hat. Hiezu wird das mikroskopische Bild des Objektes auf den Spalt eines entsprechenden Spektrographen projiziert. Gebraucht man nun eine Lichtquelle wie die Wasserstofflampe, die ein kontinuierliches Spektrum gibt, so erhält man ein Absorptionsspektrum für alle Wellenlängen und kann die Feinstruktur dieses Spektrums untersuchen.

Experimentelle Einzelheiten über die verwendeten Methoden und Angaben über die Art der zu erwartenden Ergebnisse werden angeführt. Die Methode wurde auf eine ganze Reihe von verwie mit nicht polarisiertem Licht. Die bisher benutzten Wellenlängen erstreckten sich von unter 240 mμ bis zu 14 μ.

REFERENCES

R. BARER, *Brit. Sci. News*, 1 (1948) No. 6, p. 12.
R. BARER, *Lancet*, 1 (1949) 533.
R. BARER, in *Cytology and Cell Physiology*, Ed. G. Bourne, 2nd Ed. Oxford 1950, Clarendon Press.
R. BARER, A. R. H. COLE, AND H. W. THOMPSON, *Nature*, 163 (1949) 198.
A. BOUWERS, *Achievements in Optics*, New York, Amsterdam 1946, Elsevier.
W. R. BRODE, *Chemical Spectroscopy*, 2nd Edn. New York 1943; John Wiley & Sons Inc.
G. L. BROWN AND J. T. RANDALL, *Nature*, 163 (1949) 209.
C. R. BURCH, *Proc. Phys. Soc. London*, 59 (1947) 41.
T. CASPERSSON, *Skand. Arch. Physiol.*, 73 (1936), suppl. 8.
T. CASPERSSON, *J. Roy. micr. Soc.*, 60 (1940) 608.
T. CASPERSSON, in *S.E.B. Symposia* No. 1, Cambridge 1947, University Press.
P. A. COLE AND F. S. BRACKETT, *Rev. Sci. Inst.*, 11 (1940) 419.
C. H. DIEKE, in *Instruments and Measurements Conference Transactions*, Stockholm 1947, p. 17.

G. Z. DIMITROFF AND J. G. BAKER, *Telescopes and Accessories*, Philadelphia 1945, Blakiston.
I. GERSH AND R. F. BAKER, *J. cell. comp. Physiol.*, 21 (1943) 213.
E. R. HOLIDAY, *J. Sci. Inst.*, 14 (1937) 166.
E. R. HOLIDAY AND E. M. JOPE, (1949) in preparation.
H. H. HOPKINS, (1949) personal communication.
E. E. JELLEY, *J. Roy. micr. Soc.*, 56 (1936) 101.
E. M. JOPE, in *Haemoglobin*, Ed. F. J. W. Roughton and J. C. Kendrew, London 1949, Butterworth.
J. R. LOOFBOUROW, J. F. SCOTT, AND R. L. SINSHEIMER, *Science*, 107 (1948) 302.
A. W. POLLISTER AND H. RIS, *Cold Spring Harbor Symposia*, XII (1947) 147.
Lord RAYLEIGH, *Collected Papers*, Vol. III (1902) 47.
G. SCHEIBE, *Z. angew. Chem.*, 42 (1929) 1017.
G. SCHEIBE AND A. NEUHAUSSER, *Z. angew. Chem.*, 41 (1928) 1218.
B. THORELL, *Acta med. Scand.*, suppl. CC (1947).
F. TWYMAN AND F. SIMEON, *Trans. opt. Soc.*, 31 (1930) 169.

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