SOURCES OF ERROR IN TWO-WAVELENGTH MICROSPECTROPHOTOMETRY

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Summary

This paper is concerned with defining the conditions under which the two-wavelength method can be used accurately and precisely for studies of Feulgen-stained sections.

A microspectrophotometer suitable for such measurements is described. It incorporates a precise photometric field diaphragm, a wavelength reference source, and makes provision for both direct and monochromator illumination.

Theoretical calculations indicate that non-specific light loss (scattering) causes extinction-dependent errors of considerable magnitude when the reference background used is a blank slide but not when cytoplasm is used. Methods of compensating this error are described. It is shown that differences in the scattering properties of stained and unstained nuclei and of nucleus and cytoplasm are negligible. A photometric method of matching refractive index of section and mountant is described.

Very accurate wavelength determination and maintenance is required. The second wavelength, at which the object being studied has half the extinction of the first wavelength, must be maintained with a precision of $\pm 1 \cdot 5$ Å to avoid day to day variations greater than $\pm 1\%$. The second wavelength must be determined experimentally with an accuracy of ± 1 mμ if errors between nuclei of extreme extinctions are to be kept below $\pm 1\%$. At least six nuclei must be used in any such determination and glare errors should be compensated.

Glare causes a systematic error in two-wavelength measurements, and an extinction-dependent error in central-plug methods. The latter method is very sensitive to focus while in the former considerable focal latitude is permissible.

Considerable errors can result if the illumination or photometric response of the area in which the object is lying, or both, differ from that of the area surrounding the object but which is still in the photometric field.

The amount of chromophore present in the cytoplasm is rather high and can cause serious errors especially when a non-section background is used in the two-wavelength measurements. Methods are described for compensation for this error and a comparison is made between the two-wavelength and central-plug methods with respect to cytoplasmic chromophore errors.

When properly used the method is quite adequate to detect small percentage differences between different kinds of nuclei.

I. INTRODUCTION

Three types of methods are used in microspectrophotometry—single wavelength methods involving only one reading on the object being studied, the scanning method, and the two-wavelength method.

The first method, which has been the most popular, is suitable only for objects of a regular shape, a fact which greatly restricts its utility. When used to measure

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spherical objects such as nuclei it usually makes assumptions about the distribution of the absorbing substance within the object. In the application of the "central-plug method", for example, it is assumed that the chromophore has substantially the same concentration throughout the volume of the nucleus, a situation which, in the experience of this laboratory, is rather rare. In addition to these disadvantages the method is subject to distributional error, to the effects of glare (which are related to the extinction of the object being measured), and to out-of-focus effects which are also extinction-dependent. A full discussion of this method has been presented by Davies and Walker (1953), Pollister and Ornstein (1955), Swift and Rasch (1956), and Leuchtenberger (1958).

The scanning method, in which the photometric field is scanned by a small aperture, is suitable for objects of any shape and is free from distributional error provided the scanning aperture is small enough. It is, however, subject to glare and out-of-focus errors. Not all laboratories are able adequately to test and service the rather complex electronics necessary in the apparatus.

The two wavelength method of Ornstein (1952) and Patau (1952) is free from distributional error, out-of-focus errors (Mendelsohn 1958*a*), and is subject only to a systematic rather than an extinction-dependent glare error (Howling and Fitzgerald 1959). It is suitable for objects of any shape and the electronics is no more complex than that required for the single wavelength–single reading method, although the electrical stability of the system needs to be better. On present indications, therefore, it appears to be a very valuable method.

Recent work in this laboratory has been concerned with the question of the constancy of deoxyribonucleic acid (DNA) in normal nuclei. Although the DNA-constancy hypothesis has been widely accepted (see, for example, Vendrely 1955 and Mirsky and Osawa 1961) critical study of the data at present available does not entirely support the concept of absolute constancy. It is clear that complete confidence in the microspectrophotometric method being used is necessary if small percentage differences in the DNA content of different types of nuclei are to be detected and validated. The aim of the present paper is to examine the two-wavelength method in detail in order to determine whether it is suitable for this type of study.

II. Materials and Methods

(*a*) *Histological Material*

Unless otherwise stated the materials used in the present study were paraffin sections, 15 μ thick, of formalin-fixed rabbit liver which had been stained by the Feulgen procedure of de Tomasi (1936).

(*b*) *The Microspectrophotometer* (*see Plate 1*)

The machine, which was designed not only for central-plug and two-wavelength microspectrophotometry but also for measurements on dark-ground scattering and for traverse scanning of fields with small apertures in bright-field, interference, and polarization microscope images, is described below.

A ribbon-filament tungsten lamp run from a voltage stabilizer was the primary light source; for two-wavelength work it was usually run at about half maximum

voltage. A standard wavelength source (Siemens' Sieray M2 low-pressure mercury arc) was fitted on a track just in front of the tungsten source in such a way that it could automatically be positioned coaxially with the tungsten source. The lamp housing of this source also served as a secondary shutter for testing the intrusion of room light, etc. into the photometric axis.

The tungsten source was focused on the entrance slit of a Hilger D275 (glass prism) monochromator by means of a Watson Conradi condenser. Between the condenser and the monochromator was a hinged mirror which, in conjunction with another hinged mirror situated near the microscope, was able to by-pass the monochromator and provide direct illumination of the microscope field. The mirrors were mechanically coupled and were actuated by a rod coming to the front of the instrument. A suitable condensing lens and filter system was situated between the two mirrors. Over the exit slit of the monochromator one of a number of apertures varying from 0·4–2·0 mm diameter was fitted in order to limit the length, and sometimes the width, of the exit slit.

The microscope was a Leitz Ortholux fitted with the photo-ocular attachment. The condenser provided with the instrument was not achromatized and it was found that small percentage differences in the apparent DNA content of nuclei, determined by the two-wavelength method, resulted when the condenser was focused at one or other of the two wavelengths selected. This difference was presumed to result from uneven illumination of the field at the wavelength which was out of focus. Except for centring the projected image, where a high level of illumination was required, this condenser was replaced by a 16-mm achromatic objective of numerical aperture (N.A.) 0·25. The image of the exit slit of the monochromator was focused in the plane of the specimen by this objective. The size of the exit slit or its apertures thus determined the area of field illuminated. For the measurements on nuclei a slit width of 0·6 mm was used. This corresponds to a waveband of 2 mμ.

The binocular part of the photo-ocular was used for visual inspection of the field (one side) and for camera lucida drawing of the selected object (the other side). The camera lucida (Leitz) gave a magnification of 4000 at the drawing paper, being fitted with a $\times 20$ ocular. The inspecting ocular was fitted with a graticule consisting of a pair of cross-lines and a series of concentric rings.

The normally photographic part of the photo-ocular projected the microscope image into a light-tight, internally blackened, 3-in. diameter brass tube (projection tube) containing a right-angle bend about 3 in. above the ocular. A front surface mirror was mounted on a universal joint in the centre of this bend. This mirror could be tilted in two planes by means of universally jointed fine screws which passed through the wall of the projection tube; they were fitted with lock nuts. The mirror-tilting mechanism permitted a very exact centring of the image projected on the photocell with that seen in the inspection ocular of the microscope. The centring proved very stable, seldom moving more than 1 or 2 mm (at the photocell) over a period of days.

The horizontal part of the projection tube was fitted with two baffles to eliminate internal reflection. A narrow-diameter tube (fitted with a light-tight cap) was let into it at an angle of 20° near the photocell diaphragm end. This tube was used for

viewing the projected image during the centring operation. At the end of the projection tube was attached the photocell diaphragm and the centring–traversing screen assembly.

The photocell diaphragm was a microscope condenser diaphragm opening to a maximum of 30 mm, equivalent to 15 μ at the object plane. The actuating lever of the diaphragm was replaced by a silver-steel rod (to avoid bending) which was attached to a collar of diameter 3·25 in. A movement of this collar of 1 cm was equivalent to a diaphragm opening of about 5 mm. A fine piano-wire pointer attached to the collar moved over a scale graduated in units approximately equivalent to a 1-mm diaphragm opening; each unit was further graduated in tenths. The pointer and scale were inspected by a $\times$ 10 magnifying glass and were illuminated by a low-wattage bulb. With this arrangement there was virtually no error in setting the diaphragm openings ($<0{\cdot}25\%$). The diaphragm itself was calibrated directly for each division of the collar scale by measuring the aperture along eight equidistant diameters with a dissecting microscope fitted with ocular graticules. The exact areas of the diaphragm openings at any setting of the collar scale were thus determined.

The centring–traversing screen assembly consisted of a light-tight slide situated immediately in front of the photocell diaphragm in which either a centring screen or a traversing screen could be mounted. The former was a brass plate into a depression in which was mounted a piece of Bristol board ruled with concentric circles and a pair of cross-lines similar to those in the viewing ocular graticule. When the screen was pushed down to its lowest extent the cross-line intersection was exactly at the centre of the photocell diaphragm; when pulled up, the screen was above the outer limit of the diaphragm. The traversing screen was a brass plate in which was drilled a series of apertures of various sizes centrally placed with respect to the photocell diaphragm. The plate was moved across the projected microscope image by means of a graduated screw. By selection of a suitable screen aperture the light intensity at any given point along a traverse of the projected image could be determined. Since the whole assembly could be rotated around a graduated scale, any point in the projected image was accessible.

The photocell was an EMI 9558B (tri-alkali type) of cathode diameter 44 mm, a tube with high sensitivity (max. 2000 μA per lumen) and low, stable, dark current (max. 0·05 μA). An opal glass diffusing screen was mounted in front of the photocell. The high-tension supply was a Dynatron Ltd. type N103.

The output from the photocell was read directly, usually on a Microva AL4 galvanometer of sensitivity 2 μA full scale. For measurements at low light intensities or fluxes a Cambridge galvanometer of 1 μA full scale was used; this galvanometer was adjusted to have a period of 15 sec in order to smooth out the fluctuations in output of the photocell which are inevitable when the cell is operating near its maximum sensitivity.

(*c*) *Method of Operation of Microspectrophotometer*

The centring of the projected image was checked daily as described above using the condenser supplied with the microscope and a slide containing carbon particles as the object.

After changing to the 0·25 N.A. achromatic condenser the wavelength setting was checked. This can be done to an accuracy of 1 Å as follows. The tungsten-source image of the monochromator exit slit is accurately focused with a 16-mm objective using a monochromator slit of 0·3 mm. The mercury source is pulled into position and the mercury yellow lines located by adjustment of the wavelength drum. At narrow slit openings a very sharp black line is present between the two coloured mercury lines. This black line is centred in the image of the exit slit (visible in the microscope) with the wavelength drum. The wavelength is now read off, using a magnifying glass. The correct value was taken to be 5780 Å. Once a few loose components had been located and the lubrication renewed, the monochromator proved reasonably stable. The maximum deviations found over an extended period were ± 2 Å, and these seemed most likely to be due to temperature fluctuations.

The wavelength drum on the monochromator was fitted with two adjustable stops, one for each of the two chosen wavelengths. If the standard wavelength deviated from 5780 Å the stops on the wavelength drum were adjusted accordingly.

A nucleus suitable for measurement was located under a 2-mm apochromatic objective. After centring in the inspection ocular the nucleus was drawn with the camera lucida and its maximum diameter determined in millimetres. Reference to a previously constructed table showed the photocell diaphragm opening corresponding to nuclear diameter plus 40%, and the relative area of such a diaphragm opening (square of the diaphragm radius in microns at the object plane).

Transmittances of nucleus plus surround were then determined at the chosen wavelengths, an area of slide not containing section being used as the reference background. The transmittances were calculated from the mean of two intensity measurements which differed from one another by no more than 0·4% of a full-scale deflection. A third intensity measurement was made if a greater difference was found. This was only rarely necessary.

The transmittances were converted into multiplication factors using Mendelsohn's tables (1958*b*), the chromophore content being the product of this factor ($\times 10^{-3}$) and the relative area.

(*d*) *Theoretical Calculations*

Scanning by the traversing gear of the microspectrophotometer indicated that the nuclei examined in the present study showed little variation in extinction across their projected area. In making theoretical calculations, the nuclei can, therefore, be considered equivalent to flat plates.

The light flux measured by the galvanometer will be the sum of the light passing through the nucleus and the light passing through the area surrounding the nucleus. The transmittances measured at the two wavelengths will thus be:

$$T_1 = (T_o A_o I_n + A_b I_b)/A_t I_m \ , \tag{1}$$

and

$$T_2 = (T_o' A_o I_n + A_b I_b)/A_t I_m \ , \tag{2}$$

where T_1 and T_2 are the transmittances at the two wavelengths chosen for the two-wavelength method; T_o and T'_o are the two transmittances of the object and are related to one another in such a way that

$$T_o = 1/\text{antilog } E_1 \ ,$$

and

$$T'_o = 1/\text{antilog } E_2 \ ,$$

where E_1 and E_2 are two extinction values related in such a way that $E_1 = 2E_2$; A_o is the area of the object; A_b is the area of the background surrounding the object; and A_t is the total area enclosed by the photocell diaphragm; I_n is the intensity of the light illuminating the object; I_b the intensity illuminating the background; and I_m either the mean intensity over the whole illuminated area (uneven illumination) or the intensity of the unattenuated reference background (other cases); normally $I_n = I_b = I_m$. In calculations involving variations in the sensitivity of various areas of the photocell cathode I_n and I_b represent the relative sensitivities of the areas of the photocell on which the object and background respectively fall. I_m in this case is the mean sensitivity of the total photometric field.

In the theoretical study of the method, suitable values were substituted in equations (1) and (2) to give a pair of transmittances (T_1 and T_2) which can then be entered in Mendelsohn's tables and the appropriate multiplication factor read off. In the work reported below this multiplication factor provided the basis for determining what effect certain changes in conditions would have on the apparent chromophore content of objects studied by the two-wavelength method.

III. Study of the Photometric Method

(*a*) *Non-specific Light Loss*

When the transmittances of objects under study are determined with a reference background beyond the section it is clear that any non-specific light loss (scattering) in the section will cause a spurious decrease in the transmittance.

The effect of this factor may be calculated from equations (1) and (2). To do this the values of I_n and I_b must be multiplied by $(1-S)$, where S is the non-specific light loss $[1-(I/I_o)]$. In such calculations it is assumed that the light loss is the same in the object being measured and the material surrounding it which is included in the photometric field.

The results of such calculations are shown in Figure 1. It will be seen that large errors result even when the non-specific light loss is low. The error is inversely proportional to the extinction of the object being measured [compare curves (*a*)] and directly related to the relative area of the background surrounding the object [compare curves (*a*) and (*b*); (*c*) and (*d*)].

In order to ascertain the magnitude of the errors likely to arise under ordinary circumstances, determinations of non-specific light loss were made on a section

processed by the normal Feulgen procedure but with the basic fuchsin omitted from the Schiff reagent. This showed the non-specific light loss to be approximately 0·8% when the section was mounted in a medium of matching refractive index.

This determination, however, tells little about the possible non-specific light loss from *stained* nuclei at the wavelengths used in the actual determinations where

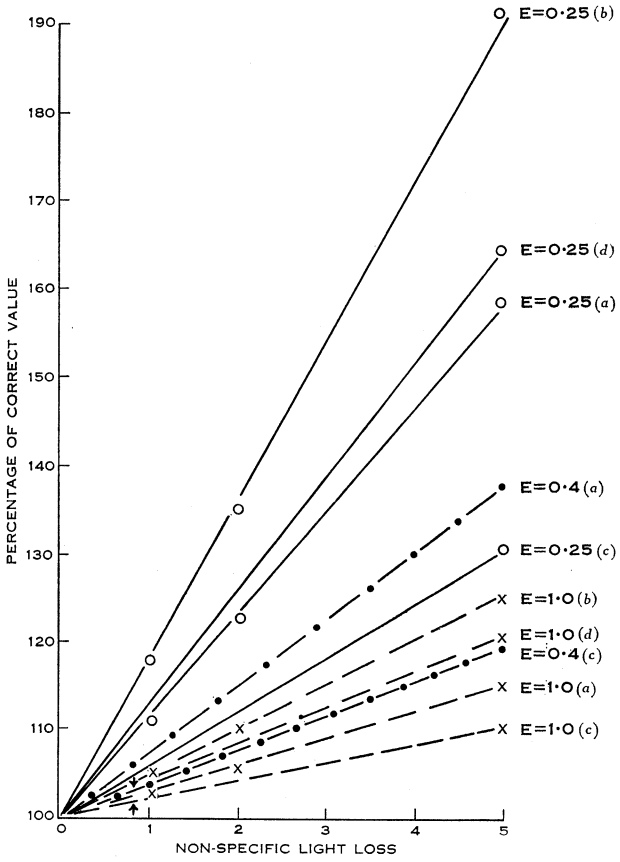

Fig. 1.—Effect of non-specific light loss, expressed as a percentage of the total incident intensity, on the apparent amount of chromophore in an object. Curves (*a*) and (*b*) at each extinction refer to situations where the area of the background is the same as that of the object and twice that of the object respectively, i.e. the total area measured was twice and three times that of the object, respectively; the object is assumed to have the same level of light loss as the background. Curves (*c*) and (*d*) refer to similar cases but the object is assumed to suffer no non-specific light loss. An area beyond the section served as reference background in each case.

anomalous dispersion may well influence the picture. It is possible to obtain information about light loss in stained sections by an indirect method which is described below. This method depends on the fact that, in the presence of scattering, there is a change in the ratio of E_2 to E_1.

Let the non-specific light loss be expressed as an extinction E_s which is equal to $-\log(1-S)$. The apparent extinction E_a will thus be

$$E_a = E+E_s,$$

where E is the true extinction. Let the ratio between the true extinctions E' and E at two chosen wavelengths be r. The observed ratio (R) between the apparent extinctions (E'_a and E_a) will be

$$R = \frac{E'_a}{E_a} = \frac{rE+E_s}{E_a} = \frac{r(E_a-E_s)+E_s}{E_a} = \frac{E_s(1-r)}{E_a} + r. \tag{3}$$

If R_1 is the ratio in a second nucleus with extinctions E'_{1a} and E_{1a} then

$$R-R_1 = E_s(1-r)\left(\frac{1}{E_a} - \frac{1}{E_{1a}}\right), \tag{4}$$

and

$$E_s = \frac{(R-R_1)E_aE_{1a}}{(1-r)(E_{1a}-E_a)}. \tag{5}$$

The value of S can readily be calculated from E_s, for:

$$S = 1-1/\text{antilog } E_s. \tag{6}$$

A series of over 60 liver parenchymal nuclei with central extinctions varying from 0·2 to 1·0 were studied at two wavelengths giving a ratio r of about 0·5. The actual ratio between the extinctions at the two wavelengths was determined twice on each nucleus after correction of the measured extinctions for the effect of the known amount of glare in the system. It is doubtful whether there was any real difference in ratio between the two extreme extinction groups (mean 0·25 and 0·95). The difference certainly was unlikely to have exceeded 0·005. This corresponds to an E_s [equation (5)] of 0·0034; the value of S [equation (6)] is thus 0·008 or 0·8%.

Since this value is the same as that for the processed but unstained section referred to above it, it may be concluded that anomalous dispersion is not a factor of importance under practical conditions. It also indicates that nuclei and cytoplasm scatter light to about the same extent.

Under practical conditions, of course, the two wavelengths at which the transmittance determinations are to be made are selected under conditions in which scattering is already present. Let us assume that the two wavelengths were chosen in material which scattered light to the extent of 1%, that the extinction of the nuclei on which the wavelengths were determined was 0·5, and that the background and nuclear areas were equal. These approximate the conditions in the present experiments.

Under these conditions the errors are shown in curve A in Figure 2(a). These errors are less than those calculated on the basis of an E_2/E_1 ratio of exactly 0·5 in the absence of scattering (curve B) but they are still considerable. Thus if the average nucleus has an apparent extinction of 0·5 in the presence of 1% scatter, nuclei with extinctions of 0·2 and 1·0 but with the same chromophore content as the average nucleus would have relative errors of +9% and −1% respectively in their extinction values and also in the apparent amount of chromophore contained within them.

The extinction of a nucleus is a function of its volume. It can readily be shown that, with the total amount of chromophore constant, the volume is a function of the reciprocal of the extinction raised to the power of 1·5. From this function the relation between relative nuclear volume and error shown in Figure 2(*b*) was calculated, giving the nucleus with extinction 0·5 an arbitrary volume of 1.

From this figure it can be seen that an apparent correlation between chromophore content and nuclear volume can arise from the presence of non-specific light loss.

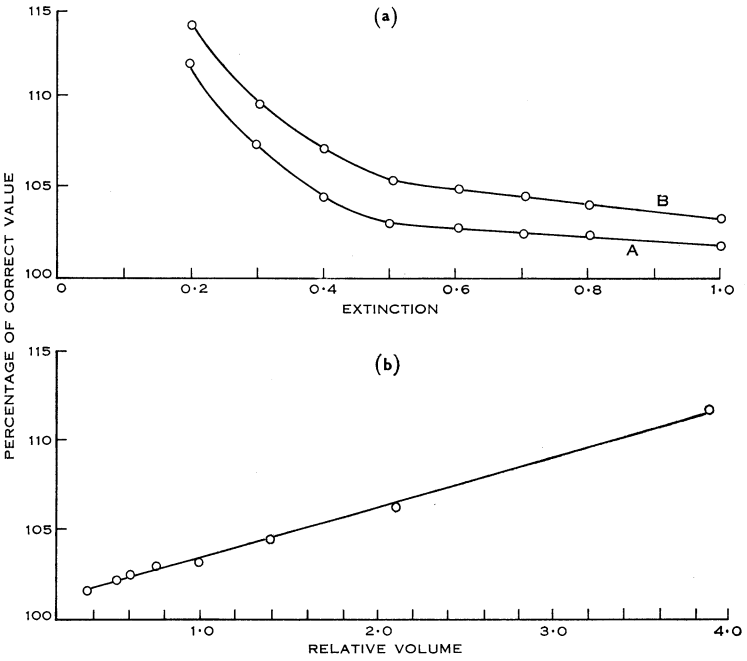

Fig. 2.—(*a*) Errors in apparent chromophore content in the presence of 1% non-specific light loss of objects of different extinctions but with the same total chromophore content. In *A*, the E_2/E_1 ratio was exactly 0·5 on objects of extinction 0·5. In *B*, the ratio was 0·5 only in the absence of any light loss. (*b*) Error related to the volume of an absorbing object in the presence of 1% non-specific light loss. The volume of an object of extinction 0·5 is given an arbitrary value of 1·0 and it is assumed that there is no change in total chromophore with volume.

The errors arising from the central-plug method (Lison) are much smaller than those arising from the two-wavelength method with non-section reference background being used in each case. Thus a nucleus of extinction 0·25 would have, in the presence of 1% scatter, an error of about 2% in the central-plug method and a little over 10% in the two-wavelength method.

If, as appears from the above experiments, nucleus and cytoplasm have essentially the same scattering properties, neither method would be subject to light-loss error when cytoplasm was used as the reference background.

It is clear from these results that a very close matching of the refractive indices of section and mountant is desirable in order to keep light scattering loss by the section to the minimum. If the non-specific light loss is known and is the same for object and background it may be allowed for by multiplying the experimentally determined values of T_1 and T_2 by $1/(1-S)$.

(*b*) *Method of Matching Refractive Indices*

Accurate matching of the mean refractive index of the tissue section and that of the mounting medium was made by a photometric method.

Using direct (non-monochromator) illumination of standard intensity, a condenser giving dark ground illumination (Leitz phase contrast) and a 16-mm objective, measurements of the difference in light intensity between blank field and section were made with the section mounted in media of different refractive indices. The media were mixtures of paraffin oil and 1-bromonaphthalene and their refractive indices were determined with an Abbe refractometer.

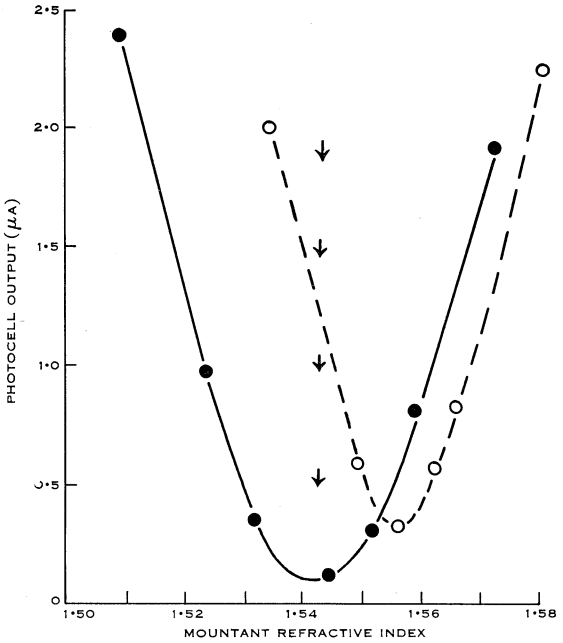

Fig. 3.—Effects of refractive index of the mounting medium on the intensity of dark-ground scattering by liver sections. The arrows show the axis of symmetry of the solid curve.

The symmetrical curve resulting from plotting mountant refractive index and intensity of dark-ground scattering is shown in Figure 3. It will be seen that the minimum in the curve is clearly defined, especially when the midpoint between corresponding points on the arms of the curve (arrows) is used for its determination. The slope of the curve is a function of the intensity of the illumination used. The dotted curve in Figure 3 is from an experiment on another section in which the light

intensity had been increased. Complete curves have been run on some half dozen sections from various organs and have shown matching refractive indices of from 1·543 to 1·570.

The mounting medium seems to need changing daily because of an apparent change in refractive index with time; this is presumably because of evaporation of the bromonaphthalene.

Some latitude in the refractive index matching is possible. By measuring a set of nine standard nuclei (mean extinction about 0·5) mounted successively in three media differing by −0·01, 0, and +0·01 from the optimum refractive index as determined above, it was shown that the change in apparent DNA content of liver nuclei in the first and third media was about +3%.

(c) *Wavelength Setting*

(i) *Choice of the Two Wavelengths*

Eleven complete absorption curves, taken from the central regions of homogeneously stained liver parenchymal nuclei with extinctions ranging from 0·213–1·035, were constructed. The flat absorption maximum lay at 555–565 mμ in all cases. The wavelength 565 mμ was used throughout the measurements as the one giving the higher of the two extinctions.

For the second wavelength, to give an extinction half that at 565 mμ, a choice may be made between the low and high parts of the wavelength range. The present choice was for the one lying at the higher wavelength because it was more convenient in terms of placing mechanical stops on the wavelength drum and because it seemed more consistent than the one lying at lower wavelengths, even when the greater slope of the curve at the higher wavelengths is taken into consideration.

The final value chosen for the second wavelength was 595·0 mμ. Other sections prepared with Schiff reagents made with a different batch of basic fuchsin gave a second wavelength of 603·7 mμ.

(ii) *Importance of Wavelength Setting (Ratio Error)*

Theoretical calculations relating to the importance of accurate wavelength setting were made as described above. Values of T_o and T'_o were calculated for various ratios of E_2/E_1 over the range of 0·4–0·6 and these values were then substituted in equations (1) and (2). In most cases A_o was made equal to A_b. The results are shown in Figure 4. For purposes of discussion the errors resulting from wrong choice of wavelengths can be considered to be of two types—a general and an extinction-dependent error.

The *general error* is considerable, averaging about ±3% for a change of 0·01 in the ratio between E_2 and E_1 (negative when less than 0·5, positive when greater). Figure 4, inset *A*, shows the relation between the E_2/E_1 ratio and the wavelength as determined on homogeneously stained liver nuclei with extinctions of about 0·7. It will be seen that a change of ratio of 0·01 corresponds to a wavelength change of about 0·46 mμ. To obtain a precision of ±1% in the readings the wavelength would have to be controlled within the limits ±1·5 Å. The method of wavelength checking used in the present work is good enough to give this precision.

The general error would not, by itself, be very significant. Provided the wavelengths were kept constant it would lead to a *systematic* error only.

Inspection of Figure 4 shows that an object with an extinction of 0·25 has a greater positive error than an object of extinction 1·0 when E_2/E_1 is greater than 0·5 and a greater negative error when E_2/E_1 is less than 0·5. It is this error-difference

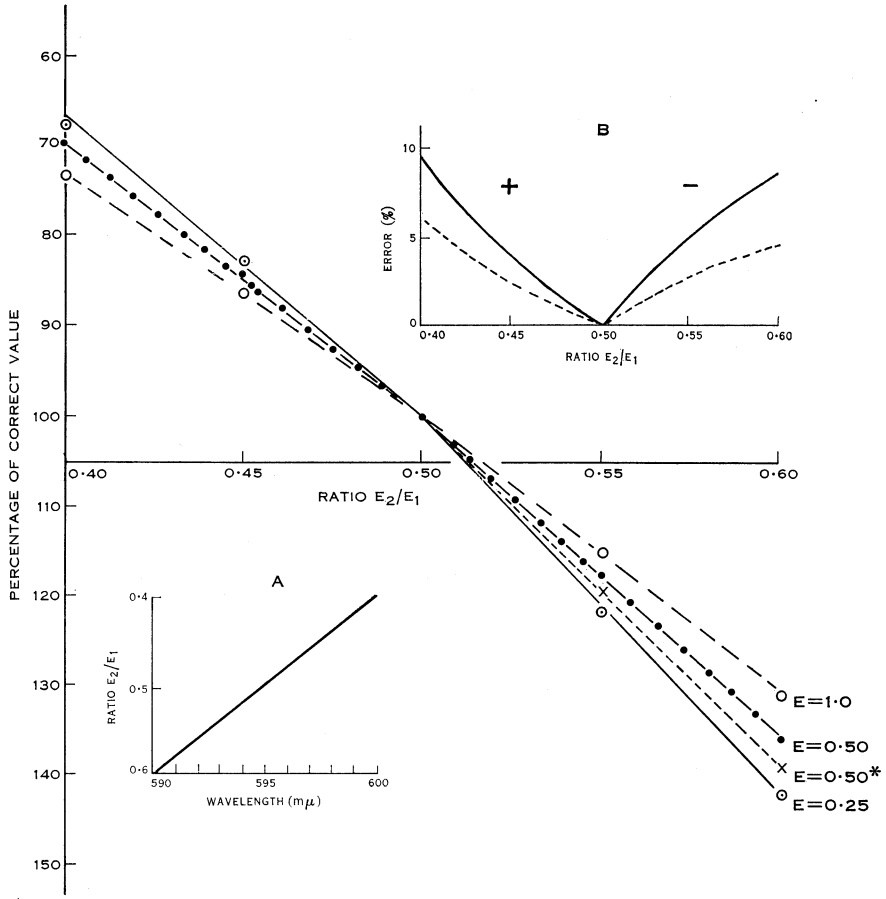

Fig. 4.—Effect of two-wavelength ratio on the apparent amount of chromophore in an object. Inset *A* shows the change of ratio with wavelength. Inset *B* shows the level of extinction-dependent error between objects of extinctions 1·0 and 0·25 (solid line) and 1·0 and 0·4 (broken line).

to which the term *extinction-dependent error* has been given. It may be expressed quantitatively as: percentage of correct value at $E = 1{\cdot}0$ minus percentage of correct value at $E = 0{\cdot}25$ divided by percentage of correct value at $E = 0{\cdot}25$. This expression has been graphed as the unbroken line in Figure 4, inset *B*, where the error is expressed as a percentage.

The extinctions of most of the nuclei measured in the rabbit liver section ranged from about 0·4–1·0. The error which could occur with these two extreme extinctions is shown approximately by the broken line in Figure 4, inset *B*.

In order to provide a practical check for these theoretical calculations readings were made on fowl blood cells. The wavelength settings necessary to give various values of E_2/E_1 over the range 0·4–0·6 (0·25 intervals) were first determined on "smeared" red cell nuclei, which are very homogeneous. Estimations of the apparent chromophore content of a lymphocyte with an extinction of 0·16 and a red cell with an extinction of 0·75 (both extinctions corrected for distributional error) were then made by the two-wavelength method at various E_2/E_1 ratios. The values were expressed as a percentage of the value obtained when E_2/E_1 was 0·5. The points lay on straight lines and the magnitude of the error was virtually identical with the theoretically calculated error.

It should be pointed out that the ratio error is also to some extent dependent on the amount of background surrounding the object. This is shown by the curve in Figure 4 marked with an asterisk which represents an object of extinction 0·5 surrounded by twice the normal area of background ($A_b = 2A_n$).

(iii) *Determining the Second Wavelength*

It is apparent from Figure 4, inset *B*, that, in order to keep the extinction-dependent error down to $\pm 1\%$, the ratio E_2/E_1 must lie in the range 0·48–0·52 which corresponds to a wavelength range of about ± 1 mμ.

Nuclei show quite a range of ratios in their extinctions at two fixed wavelengths. Glare-corrected extinction values were determined for reasonably homogeneous central areas of 60 liver parenchymal nuclei at wavelengths of 565 and 595 mμ and the ratio E_{595}/E_{565} calculated. Only 60% of the ratios lay within $\pm 0{\cdot}02$ of the mean while 90% lay within $\pm 0{\cdot}04$ of the mean. The standard deviation of the ratio is about 0·025 which indicates that about six determinations of the second wavelength are required to obtain a mean value of sufficient precision.

The mean value is, however, virtually independent of the extinction of the nuclei being measured (see above) and has been found to be the same for rat adrenal cortical nuclei, fowl red cell nuclear smears, rat luteal nuclei, human leucocyte nuclei, and rabbit liver parenchymal nuclei treated with the same batch of Schiff reagent. The mean value appears to depend principally on the batch of basic fuchsin used to prepare the Schiff reagent.

(*d*) *Effect of Glare*

Theoretical study of the effect of glare on the apparent chromophore content of objects measured by the two-wavelength method was made as described above. In the basic equations the values of T_o and T'_o were increased by G and the values of I_b and I_m multiplied by $(1+G)$, where G is the glare.

The amount of glare actually present in the microscope field was measured by determining the transmittance of opaque carbon particles of about the same size as nuclei. If this transmittance is T_c then:

$$T_c = G/(1+G),$$

and

$$G = T_c/(1-T_c). \tag{7}$$

A theoretical and practical comparison of the error arising from the effects of glare on the central-plug and two-wavelength methods is shown in Figure 5. In the theoretical calculations for the central-plug method the transmittances (T') in the presence of glare were calculated as follows:

$$T' = (T+G)/(1+G). \tag{8}$$

A good agreement between the theoretical and experimentally determined errors is evident in Figure 5. In the experimental determinations the extinction at zero glare was obtained by extrapolating the curves relating extinction or Mendelsohn factor to the glare. The amount of glare was regulated by changing the illuminating system.

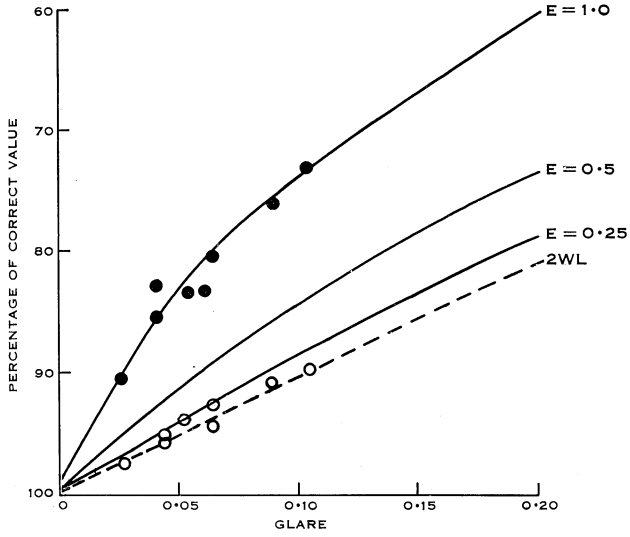

Fig. 5.—Effect of glare on the apparent chromophore content of objects of various extinctions (0·25, 0·5, 1·0) measured by the central-plug method and objects of any extinction measured by the two-wavelength method ($2WL$).

The superiority of the two-wavelength over the central-plug method is clearly evident. The former is subject only to a systematic glare error, not an extinction-dependent one as is the case with the latter.

(e) *Out-of-focus Effects*

In Figure 6 a comparison is made between the two-wavelength and central-plug methods when a lymphocyte nucleus of extinction 1·10 and diameter about 4 μ was studied at different levels of focus.

The central-plug method shows a negative error of large magnitude on either side of correct focus. Indeed the method is so sensitive to focus that it was able to pick up a difference of about 0·6 μ between the best visual focus and the best photometric focus. The two-wavelength method, on the other hand, shows only small positive

percentage errors even as far as 6 μ from correct focus. In fairness to the plug method it should be pointed out that the out-of-focus errors would be less in nuclei of smaller extinction (Davies and Walker 1953).

(*f*) *Effect of Uneven Photometric Field*

The photometric field may be uneven because of uneven illumination or because of an uneven sensitivity of the photocell cathode or for both reasons. When our microspectrophotometer was commissioned it was tested for evenness of photometric response and found to be satisfactory provided a diffusing screen was placed in front of the cathode. A re-investigation at the end of the present series of determinations indicated that the photocell cathode now gave an uneven emission.

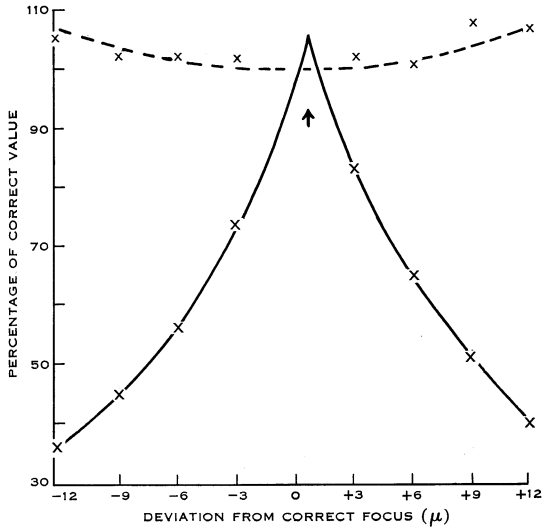

Fig. 6.—Effect of deviation from correct focus on the apparent chromophore content of a lymphocyte nucleus measured by central-plug (solid line) and two-wavelength (broken line) methods.

It is easy to appreciate that with the two-wavelength method random inhomogeneity of photocell response or of illumination within the photometric field will not cause any error. On the other hand, a difference in the level of illumination or in the photocell response between the object area and the surrounding background area will do so. The extent of the error can readily be calculated by substitution in equations (1) and (2). Under these conditions $I_n \neq I_b$, and

$$I_m = \frac{I_n A_o + I_b A_b}{A_o + A_b}.$$

In Figure 7 a plot of such calculations is shown. It will be seen that quite large errors can result from an uneven photometric field. The errors are directly related to the difference in photometric response between the object and background areas

and also to the area of the background relative to that of the object. No dependence on the extinction of the object being measured could be found.

The photometric field used in the determinations on nuclei was traversed along eight equidistant diameters and the resulting photocell emission values plotted on graph paper. The mean emission values for the area normally occupied by a nucleus was shown to differ by less than 2% from the area normally occupied by background. No significant error from unevenness of photometric response is therefore to be expected in the present series of measurements.

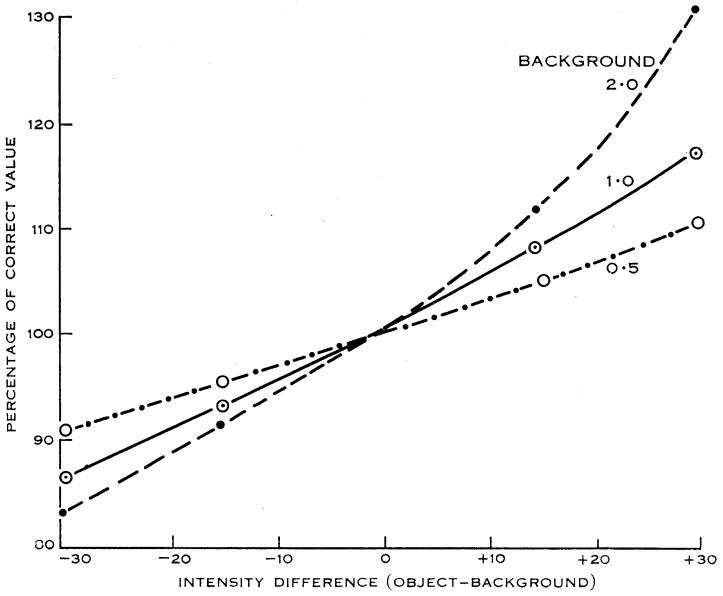

Fig. 7.—Effect of uneven photometric field (difference between object and background areas expressed as a percentage) on the apparent chromophore content of an object. The background area is expressed as a fraction of the area of the object.

(*g*) "*Cytoplasmic Chromophore*"

The term "cytoplasmic chromophore" refers to the apparent chromophore content of the cytoplasm with the present method of measurement where the reference background is an area devoid of section.

Part of the cytoplasmic chromophore is due to a true staining of the cytoplasm by Schiff's reagent even though this may not be evident to the eye; presumably the reacting substances are plasmals and oxidized lipids. The other component is a consequence of non-specific light loss. This also gives a positive error related to the size of the background area, as demonstrated above, and thus behaves similarly to true cytoplasmic chromophore.

The cytoplasmic chromophore concentration was determined experimentally by measuring the difference between the apparent chromophore contents of nuclei lying in the background areas resulting from the normally used diaphragm openings

and those lying in the background areas increased by 5–10 area units. These results indicate a cytoplasmic chromophore concentration of 0·058 arbitrary units per unit area of cytoplasm 15 μ thick (250 determinations), corresponding to 1·24 units per 1000 μ^3 for the cytoplasm immediately surrounding liver parenchymal nuclei. The standard deviation was very high.

A series of measurements was made to determine whether there was any gradient in cytoplasmic chromophore between the nucleus and the cell membrane. It was found that the concentration was essentially constant in the cytoplasm but was higher in the immediate vicinity of the membrane. This finding simplifies the determination of the chromophore concentration which can thus be done directly on cytoplasm. There was no correlation between cytoplasmic chromophore concentration and nuclear volume (60 determinations on nuclei with volumes from 85–405 volume units).

Corrections for cytoplasmic chromophore content are readily made when the nuclear volume, the area of the photometric field, and the section thickness are all expressed in microns. If A is the area of the photometric field, T is the section thickness, V the nuclear volume, C the cytoplasmic chromophore concentration (above), and M is the content of cytoplasmic chromophore then

$$M = C(AT - V) \times 10^{-3}. \tag{9}$$

That the chromogenic substance is not DNA may readily be demonstrated by digestion with crystalline deoxyribonuclease. In a 2-hr digestion, sufficient to remove over 50% of the DNA from liver parenchymal nuclei, there was absolutely no change in the content of cytoplasmic chromophore.

(*h*) *Precision of the Photometric Estimate*

On a number of occasions 10 consecutive determinations of DNA content were made on the same nucleus. The standard deviation of such measurements was usually 0·1 units in a mean of about 5·0 units. The precision (± 2 S.D.) is thus about $\pm 4\%$ for a single estimate.

A set of nine standard nuclei was measured on nine different occasions. This was done to detect any day-to-day variation in the determinations. The standard deviation of the means on different days was 0·035 units which is in good agreement with the standard error predictable from the consecutive measurements.

IV. Discussion

(*a*) *The Microspectrophotometer*

The microspectrophotometer has proved very convenient to use. Apart from the special features incorporated in it, the arrangement of the photocell diaphragm would seem to be especially advantageous. Thus Garcia (1962), working with a modification of the commonly used Pollister and Moses (1949) instrument, noted a doubling of the coefficient of variation of consecutive readings on the same nucleus when the photocell diaphragm was reset between successive determinations. In the present instrument the photocell diaphragm can be reset with an error of less than 0·25%. Garcia's overall coefficient of variation for consecutive readings on a single

nucleus was 6% while in the present case it is only 2%. Some of the improvement is no doubt due to the better electronics in the present instrument as well as the more reproducible setting of the photocell diaphragm.

(*b*) *The Two-wavelength Method*

The present study of the method has defined more closely the conditions under which it will give precise results. These conditions have proved to be much more stringent than was expected. Some of the stringency has arisen because of the use of a blank area of slide as the reference background and some is inherent in the method no matter what reference background is used.

(i) *Choice of Reference Background*

Most workers choose an area of apparently unstained cytoplasm as their reference background for Feulgen-stained nuclei, rather than an area beyond the section as has been done in the present work. The reasons for our choice of background were as follows. In sections containing small cells an area of cytoplasm completely free from nuclear chips and large enough for the two-wavelength method is not easy to find and the selected object may very easily be lost in the search for it. Such a background is, furthermore, not stable because of differences in the amounts of cytoplasmic chromophore. Further difficulties arise when making comparisons between such things as liver parenchymal nuclei, which are surrounded by much cytoplasm, and lymphocytes and Kupffer cells in the same section which lie in the liver sinusoids and have only scanty cytoplasm.

The major consequences of the choice of a non-section reference background are the presence of the extinction-dependent error due to non-specific light loss and an increase in the error due to cytoplasmic chromophore. Both of these can, however, be compensated. In the case of non-specific light loss the observed transmittances are multiplied by $1/(1-S)$ after the value of S has been determined on a section processed in the same way as the stained section but with the basic fuchsin omitted from the Schiff reagent.

It should be stressed, however, that the calculations are much easier if cytoplasm is used as the reference background. This should certainly be done wherever the nucleocytoplasmic ratio permits and where the nuclei are surrounded by similar amounts of cytoplasm.

(ii) *Cytoplasmic Chromophore*

The remaining correction for true cytoplasmic chromophore is more difficult to make because it involves a knowledge not only of the chromophore concentration but also of the nuclear volume. Nuclear volumes of irregularly shaped nuclei cannot be more than approximations but may be sufficiently accurate when the volume of included cytoplasm is large. Special problems arise in determining the chromophore concentration in the region around nuclei which lie partly or wholly in spaces such as those of Kupffer cells and lymphocytes in the liver. Taking the background reading on cytoplasm would not overcome this difficulty.

Cytoplasmic chromophore is not easy to detect microscopically in ordinary preparations. It is, however, quite evident to naked-eye examination in unhydrolysed controls. The concentration of the cytoplasmic chromophore is quite small in liver sections (about 1·2 units/1000 μ^3) when compared to the concentration of Feulgen chromophore in nuclei (about 50 units in the case of liver parenchymal nuclei).

Considerable cytoplasm is, however, of necessity included with the nucleus in two-wavelength measurements, the amount being a function of nuclear diameter and section thickness. The formula for the amount of included cytoplasm, under the present methods of measurement and for spherical nuclei, is

$$\tfrac{1}{4}\pi D^2(1\cdot 4^2 T - \tfrac{2}{3}D), \tag{10}$$

where D is the nuclear diameter and T the section thickness. Thus a nucleus of diameter 6 μ (with a volume of 113 μ^3) in a section 15 μ thick will have 718 μ^3 of cytoplasm included with it. Such a nucleus has about 5 units of Feulgen chromophore in it and the cytoplasm included with it has about 0·86 units, making a total of 5·86 units. A nucleus of 4 μ in diameter, on the other hand, with the same DNA content would have only 336 μ^3 of included cytoplasm and would appear to contain only 5·40 units of total chromophore. The difference in apparent chromophore content of two such sets of nuclei could well appear statistically significant.

Cytoplasmic chromophore is a complication not only in the two-wavelength method but also in the central-plug method, as the following considerations indicate.

Suppose the chromophore content is calculated as the product of the extinction of a small central plug and the nuclear area (Lison). Let the nuclear diameter be D and the section thickness be T. The true chromophore content of the nucleus is A and the extinction per unit path length of cytoplasm is y. When measured against blank slide as reference background the apparent chromophore content of a nucleus (A') will be

$$A' = A + \tfrac{1}{4}\pi y D^2(T-D),$$

and the error $(A'-A)/A$ will be

$$0\cdot 7854 y D^2(T-D)/A. \tag{11}$$

If the nucleus is read against a cytoplasmic reference background

$$\begin{aligned} A' &= A + \tfrac{1}{4}\pi D^2\{(T-D)y - Ty\} \\ &= A - 0\cdot 7854 D^3 y, \end{aligned}$$

and the error is

$$-(0\cdot 7854 D^3 y)/A. \tag{12}$$

It follows from equation (10) that the error in the two-wavelength method with slide as reference background is

$$+(1\cdot 96T - \tfrac{2}{3}D)(0\cdot 7854 D^2 y/A), \tag{13}$$

and when read against a cytoplasmic background is

$$-0\cdot 5236 D^3 y/A. \tag{14}$$

It is evident from these equations that when cytoplasm is used as background the error is a negative one, is independent of section thickness, and is a direct function of nuclear volume and cytoplasmic chromophore concentration. With slide as background, however, the error is a function of the difference between nuclear diameter and section thickness, of the nuclear area, and of the cytoplasmic chromophore concentration.

In Figure 8 the errors have been plotted as a percentage for nuclear diameters over the range commonly found in sections, assuming the following values: $T = 15\ \mu$ (a usual value), $A = 15$ (i.e. π times the amount found with the present two-wavelength method), and $y = 0{\cdot}001$ (a value considerably lower than is commonly found).

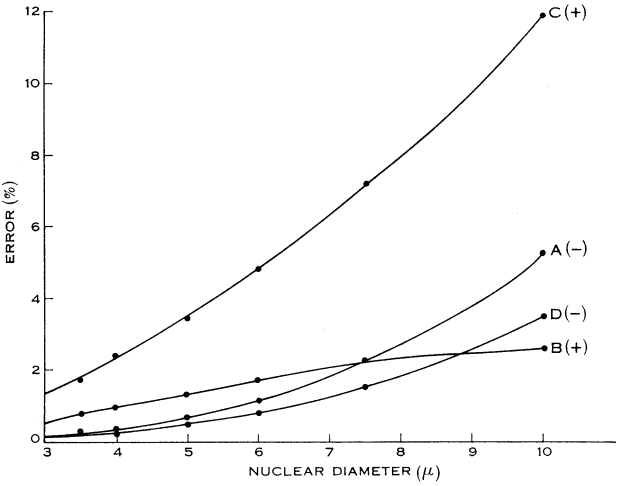

Fig. 8.—Error caused by the presence of cytoplasmic chromophore on the apparent chromophore content of nuclei of different diameter but with the same true chromophore content. For actual values of variable used in the computations see text. Curves A and B are for the central-plug method read against cytoplasm and blank slide respectively. Curves C and D are for the two-wavelength method read against blank slide and cytoplasm respectively.

It will be seen that the absolute errors are certainly not of negligible magnitude in any case. The smallest errors are given by the two-wavelength method with cytoplasm as reference background and the largest with this method using slide as reference background. With the central-plug method, slide is preferable to cytoplasm as reference background for the larger nuclear diameters although not for the smaller.

Absolute errors, however, are less important than relative errors in comparing the chromophore contents of nuclei of different sizes. The relative errors resulting in a comparison between nuclei with the same chromophore content but of diameters 3 and 10 μ are $+2{\cdot}1$ and $-5{\cdot}1\%$ for central-plug methods read against slide and cytoplasm respectively, and $+10{\cdot}6$ and $-3{\cdot}4\%$ for the two-wavelength method used similarly (Fig. 8).

It appears, therefore, that, whenever the material permits, two-wavelength readings should be made against a cytoplasmic background (when they are independent of section thickness) while central-plug measurements should be made against

non-section background and the sections should be kept as thin as possible, consistent with the nuclear diameter.

(iii) *Sources of Error*

Three potential sources of error in the two-wavelength method may operate whatever reference background is used. These are incorrect wavelength setting, unevenness of the photometric field, and glare.

The errors which result from *incorrect or inconstant wavelength setting* may reach a large magnitude. In order to keep the "general" component of this ratio error within $\pm 1\%$, the half-extinction wavelength must be controlled to within $\pm 1 \cdot 5$ Å, a precision which demands that a reference source with a readily located narrow line be incorporated in the microspectrophotometer. Correct choice of the two wavelengths to be used is also very important with respect to the extinction-dependent component of the error. If this is to be kept within $\pm 1\%$, the half-extinction wavelength, which lies on the steep slope of the absorption curve, must be within ± 1 mμ of the correct value.

Part of the stringency in wavelength control arises from our choice of the second wavelength on that part of the absorption curve lying at higher wavelengths rather than on the less steeply sloping part lying at lower wavelengths. If the second wavelength is chosen from the lower wavelength range (as recommended, for example, by Garcia 1962) the above tolerances would be multiplied by a factor of about $1 \cdot 5$. Even these tolerances place a considerable strain on the monochromator and on the method of wavelength checking.

Unevenness of the photometric field, whether due to uneven illumination or uneven photocathode response, is also an important source of error. Its magnitude is such that a difference of $\pm 10\%$ in the photometric response between the area containing the object and the background area surrounding it leads to an error about $\pm 5\%$ in the estimation of the chromophore content of the object. This error is independent of the extinction of the object but is strongly affected by the ratio between object and background areas.

This is not the only type of error to be influenced by the ratio between object and background areas. Thus the errors arising from incorrect wavelengths are also affected to some extent and the light-loss error is very strongly influenced by this ratio. Clearly the ratio should be kept constant in any series of measurements.

Glare in the two-wavelength method causes only a systematic error in the measurements, as was first pointed out by Howling and Fitzgerald (1959). Provided all measurements are made under the same conditions of glare, this factor need cause no trouble. If the value of the glare is known the error may, in any case, be compensated.

(iv) *Advantages and Disadvantages*

The present study of the two-wavelength method has confirmed and emphasized some of its advantages over the older methods using only a single wavelength. In particular the extinction-dependent glare and out-of-focus errors of the latter techniques are converted into systematic errors or are eliminated. There is no distributional error or error due to deviations from the theoretical shape of the object being measured. Very importantly, objects of any shape may be measured.

On the other hand the method requires extremely accurate selection and maintenance of the two wavelengths used in the measurements and a very accurate determination of the two transmittances.

As shown above, when the transmittances are determined against a non-section background the method is very sensitive to the effects of non-specific light loss and to the presence of cytoplasmic chromophore. When cytoplasm is used as background, however, the accuracy of the method compares favourably with that of the central-plug method.

Even when used with non-section background the method has a precision similar to that of the central-plug method. Swift and Rasch (1956) quote a series of consecutive readings on the same liver nucleus which indicates a coefficient of variation of about 2·3%; our own is 2%. A coefficient of variation of 7·5% was found by Swift (1950) for a population of mouse liver parenchymal nuclei studied by the central-plug method, and carefully selected for regularity of shape. In a series of determinations on 290 rat liver parenchymal nuclei by the two-wavelength method in this laboratory the coefficient of variation was found to be 10%. The higher value in the present case is almost certainly due to the complete lack of selection of nuclei.

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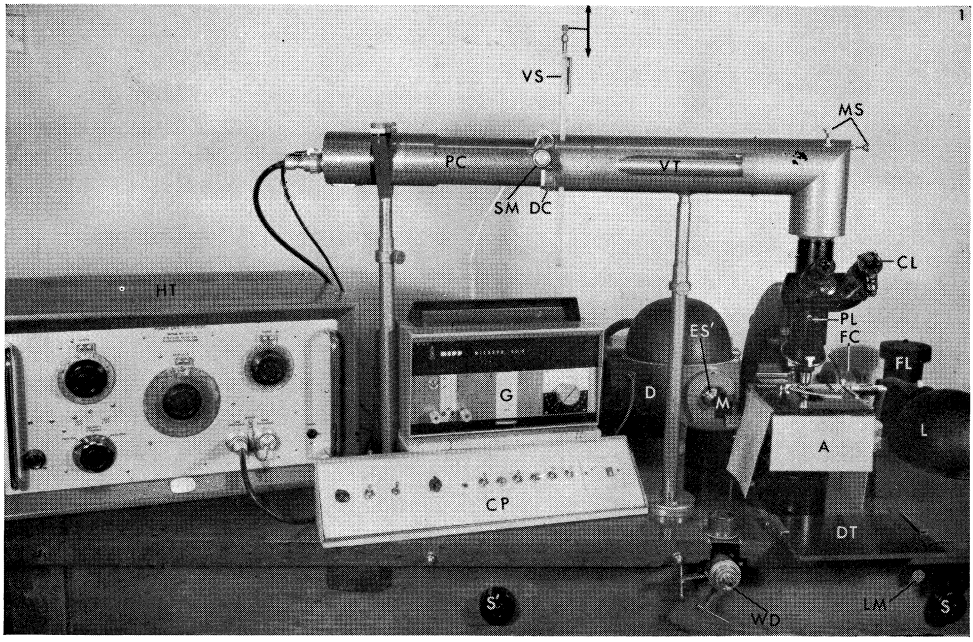
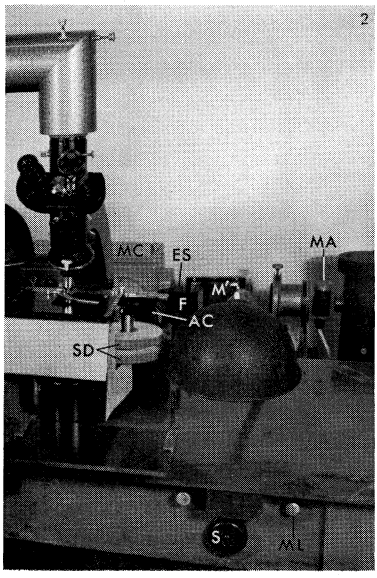
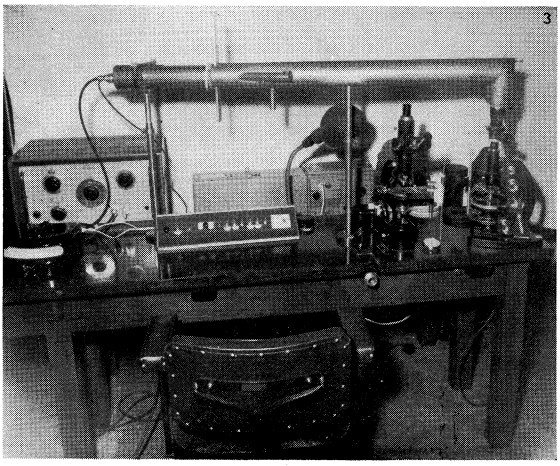
TWO-WAVELENGTH MICROSPECTROPHOTOMETRY







Explanation of Plate 1

Figures 1 and 2 show the microspectrophotometer set up for one-man operation and viewed from different angles to show all the components.

Light generated by the filament lamp (*FL*), after passing through the field condenser (*FC*), may be made to enter the entrance slit (*ES* in Fig. 2) of the monochromator (*MC* in Fig. 2) by moving the mirror lever (*LM*) or may be diverted by the mirror (*M'* in Fig. 2) through the Wratten filter (*F*) and auxiliary field condenser (*AC* in Fig. 2) to impinge on another mirror (*M*) and thus enter the microscope direct.

The monochromator is regulated by the slit assembly—the slit width being read on the drum (*D*)—and by the wavelength drum (*WD*). Note the adjustable screws fastened to the wavelength drum which act as stops to give the two chosen wavelengths. The lens above the drum helps in setting the drum to a precision of about 1 Å. The exit slit (*ES'*) is shown fitted with a centrable field lens, which has been added since the work described in this paper was done. The screws projecting out from the side of the table-top are for fitting a drive from a recorder to the wavelength drum.

The light enters a Leitz Ortholux microscope. Balsa wood disks (*SD*) have been placed over the mechanical stage controls of the microscope to improve their sensitivity. The image may be viewed through the binocular ocular assembly and drawn by means of the camera lucida (*CL*) on the drawing-table (*DT*) illuminated by the overhead lamp (*L*); the drawings are made on protocol sheets on which the galvanometer readings are also recorded. The image may be projected up the monocular phototube by pushing in the phototube lever (*PL*). Under these conditions the image is centred on the viewing screen contained in the viewing screen assembly (*VS*) by means of the mirror-adjusting screws (*MS*), the image being inspected by means of the viewing tube (*VT*).

When the viewing screen is pulled up (double-headed arrow) light passes into the photomultiplier contained in the photocell casing (*PC*). The diameter of the photocell illuminated is regulated by means of the collar (*DC*) which actuates the photocell diaphragm. Just to the left of this collar is a white calibrated diaphragm scale illuminated by a low wattage bulb supplied with current by the switch (*S'*) on the vertical face supporting the table-top. The scale is read by means of the scale magnifier (*SM*).

The photocell high tension is generated by the unit marked *HT* which is placed further back than shown (cf. Fig. 3) when the machine is used by an operator and assistant. The photocell output is read on the galvanometer *G*.

Calibration of the monochromator is done by means of the mercury arc lamp (*MA*) which is pulled into the optic axis by the lever *ML* and activated by the switch *S*. Extraneous light is prevented from entering the microscope optic axis by means of the aprons (*A*) fitted to the microscope stage. The control panel of the instrument (*CP*) contains the following switches, outlets, or controls (from right to left): mains switch, wavelength drum illuminating lamp, slit drum illuminating lamp, galvanometer lamp 1, galvanometer lamp 2, filament lamp, overhead lamps, recorder outlet, galvanometer sensitivity and period selector, galvanometer selector switch, dark current cancellation current switch, and cancellation current potentiometer. The cancellation current assembly is used for setting the dark-current zero on a Cambridge spot galvanometer, used in place of the Microva galvanometer shown, when measuring photocell outputs with polarization or interference microscopy. Current is supplied by a battery in the control panel. With these forms of microscopy a mercury or xenon arc source is used and the galvanometer period is adjusted by the switch mentioned above to smooth out any short-term variation in the lamp output.

In Figure 3 is shown the photometer set up for use with a polarization microscope. The principal modifications are: the polarizing microscope illuminated by the mercury arc and fitted with the viewing telescope from a Leitz photomicrographic apparatus, the extension of the projection tube (to give a greater magnification), the use of the traversing screen in place of the viewing screen (so that traverses across birefringent objects can be made) and the use of the Cambridge galvanometer in place of the Microva. The traversing screen fits in the viewing screen assembly (Fig. 1) and is moved by means of the screw seen at the top. The position of the screen is measured by means of the rod seen on the right-hand side of the viewing screen assembly; this rod is read against a scale cemented to the viewing screen assembly.