A "Two-Objective, One-Area" Procedure in Absorption Microphotometry and Its Application Using an Inverted Microscope

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Abstract. A 'two-objective, one-area' method and related equations are suggested to measure absorbance of microscopic stained objects. In such work, the measuring field invariably includes an image of the object and some clear area surrounding the image. The total intensity in the two areas is measured photometrically, using two different objectives, and substituted in the equation for absorbance. The equation is independent of the term representing intensity from the clear area and hence the error in the measurement of absorbance is reduced. The limitations of the 'two-objective, one-area' method are discussed and its pragmatic operation described with an experimental setup involving an inverted microscope. The method permits measurement of intensity in a part of a stained cell while the rest of the cell remains in the field of view. The method is applied to measure absorbance in Giemsa stained ascites cells and Feulgen stained liver and Human Amnion cells.

Key words: Microphotometry — Distribution error — Inverted microscope — Absorbance — Two objective one area method

Introduction

In the simplest type of absorption photometer for stained cells, an image of the cell or its part is allowed to fall on a photomultiplier and the resulting current is measured. A control reading for transmission through an area, equal to that of the cell or its part, from an empty field, is taken to give 100 percent transmission (Pearse 1972).

The measurement of cellular transmittance or absorbance by absorption photometry often involves several errors. The distributional error (d. e.) arises when there is, as in the case of biological systems (Pearse 1972), uneven distribution of absorbing material. The scanning method (Caspersson and Lomakka 1972) is, by far, the best way to minimize d. e.

In conventional absorption microphotometry the clear area within the measuring apperture, surrounding a stained specimen, sets a limit to the accuracy of measurement of absorbance. The 'two-wavelength, one area' (Garcia 1962) and 'two-area, one wavelength' (Garcia and Iorio 1966) methods are essentially meant for minimizing such error.

In this work a 'two-objective, one-area' method with related equations is suggested to measure absorbance of stained cells.

Materials and Methods

An inverted microscope, Unitron Universal Camera Microscope (Model U-13), was used in this work. The schematic diagram (Fig. 1) shows the essential components of the microscope. The original reflecting mirror was replaced with a thin surface aluminized mirror having a 'hole' at its centre and was set at the same angle 'θ' (Fig. 1) as for the original mirror.

The 'hole' was drilled vertically after arranging the mirror on a horizontal surface at the above angle. Thus, the cylindrical periphery of the 'hole' remained vertical in the final arrangement and loss of intensity because of rays striking the periphery of the 'hole' was minimal.

The rear surface of the mirror was coated with black paint to prevent transmission through minute openings, if any, on the aluminized surface. Several such mirrors with holes of diameter warying from 2 mm to 4 mm were prepared. A photomultiplier tube, connected to a photovolt amplifier (Photovolt Corporation, U.S.A., Phototube type 'B', spectral response 250 μm to 600 μm), was so arranged that its window lies just below the 'hole' in the mirror and the light transmitted through the 'hole' registers maximum photometer reading. The mirror reflects the microscopic field towards the viewing screen of the Unitron Microscope (Fig. 1). The microscope was focussed to produce a sharp image of stained cells on the screen. The 'hole' appears as a black spot (Figs 2 *A, B*).

The monochromaticity of light was ensured by moving an appropriate filter over the condenser (Fig. 1). The filter was selected after studying the absorption characteristics of the staining dye. Thus, a filter transmitting 520 $\pm$ 20 μm was suitable for the Giemsa stained ascites cells and also for the Feulgen stained Human Amnion cells, because the 50 % extinction for the two dyes occurs at this wavelength.

The field on the viewing screen could be photographed by replacing the screen with a photographic plate (ORWO, 400 ASA, 8 cm $\times$ 11 cm).

The constancy of the voltage of the lamp, illuminating the cells, was ensured by using a voltage stabilizer.

Two parfocal objectives of magnifying powers in the ratio 1 : 2 were chosen. Under proper optical alignment of the microscope, when such objectives are brought in focus over a clear (background) area of a specimen slide, the ratio of transmission through them equals inverse ratio of their magnifying powers. This is achieved by adjusting:

1) the height of the condenser above the specimen,
2) the aperture of diaphragms in the lamp housing and the condenser assembly,
3) the centring of lamp housing and the condenser assembly.

'Two-objective, one-area' procedure: Let the pair of objectives be X20 and X40. A mirror with a 'hole' of size which accomodates the image of a cell, magnified with X40 objective, is chosen (Fig. 2*A*). The voltage of the lamp is adjusted to give background reading, adjacent to the cell, of little *less than half* the full scale deflection of the photometer. The following readings are noted:

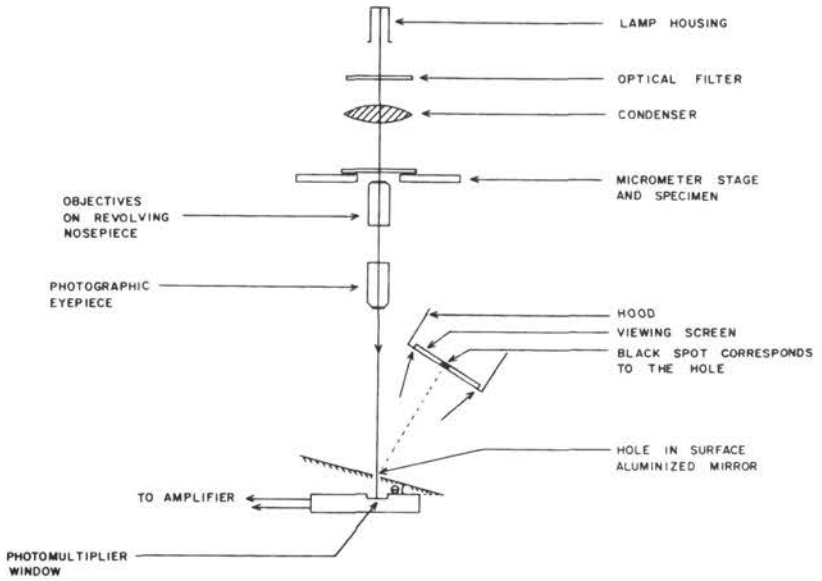

Fig. 1. A schematic diagram showing optical arrangement of an inverted microscope. A surface aluminized mirror, with a 'hole' (diameter 2 mm to 4 mm) at the centre, reflects the microscopic field towards a viewing screen. A photomultiplier below the 'hole' measures transmission through the image of a cell (see Materials and Methods) and through the background.

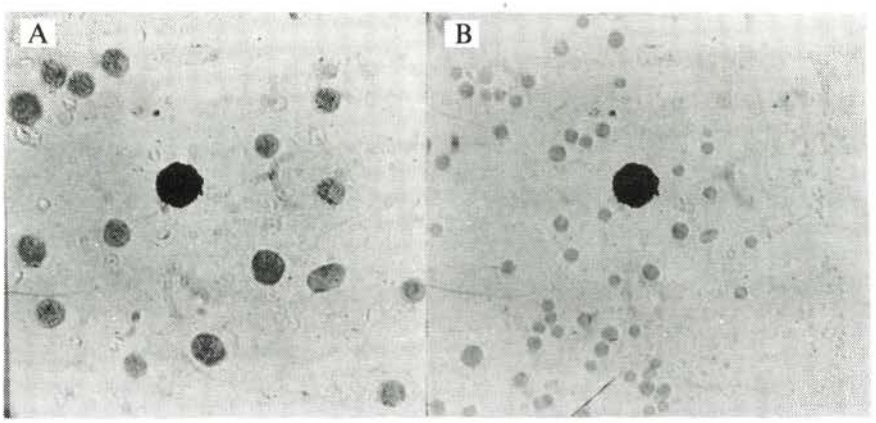

Fig. 2. Photomicrographs of Giemsa stained ascites cells with X40 (*A*) and X20 (*B*) objectives. The dark spot corresponds to the 'hole' (diameter 4 mm) in the mirror.

A) 'θ_{40}' when the image of the cell is located within the 'hole' (in practice, the image is shifted into the 'hole' until the meter registers minimum reading).
B) 'B_{40}' when the cell in 'A' is displaced and the 'hole' is covered with clear background adjacent to the cell.

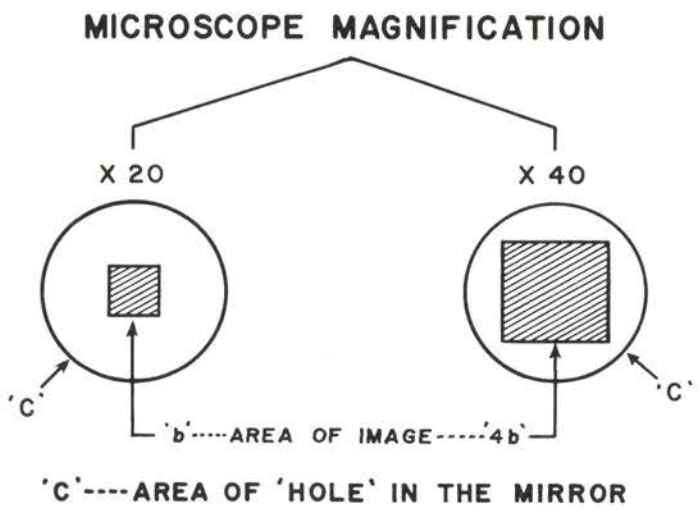

Fig. 3. The schematic diagram shows how the magnified image of the same objects will appear with X20 and X40 objectives. The circle represents the 'hole' in the mirror of Fig. 1.

Next, the nosepiece is rotated and the X40 objective is replaced with a X20 objective. The image in 'A' above will be diminished in size (Fig. 2*B*) and, if the objectives are parfocal, will continue to remain within the 'hole'. A slight displacement may be necessary to position it to give minimum photometer reading. Again, two more readings θ_{20} and B_{20} corresponding to θ_{40} and B_{40} are obtained.

From the schematic diagram of Fig. 3, the area '*c*' of the measuring 'hole' remains unchanged at both magnifications. Let the area of the image, with X20 objective, be '*b*'. Then the left over background is $(c - b)$. For the X40 objective, these two areas are $4b$ and $(c - 4b)$.

The X20 objective: The intensity transmitted through the cell and the background, surrounding the cell, is gathered by the objective and reaches the 'hole' in the mirror after it decreases as per the inverse square law. Let the intensity *per unit area* through the background, reaching the 'hole', be I_o then, with reference to Fig. 3, the transmission through the background surrounding the cell is $(c - b)I_o$. If the absorbance *per unit area* of the cell, assumed *homogeneous*, is '*a*' then the corresponding intensity *per unit area* transmitted through the cell is $I_o e^{-a}$. The total absorbance of the cell is simply the product of a and b.

Since the stained cell is in general *nonhomogeneous*, we may divide it into regions of areas $b_1, b_2 \ldots b_n$ having absorbances *per unit area* $a_1, a_2 \ldots a_n$ respectively. The transmissions through the individual regions would be $b_1 I_o e^{-a_1}, b_2 I_o e^{-a_2} \ldots b_n I_o e^{-a_n}$ and the total transmission is

$$b_1 I_o e^{-a_1} + b_2 I_o e^{-a_2} + \ldots + b_n I_o e^{-a_n} = I_o \sum_{i=1}^{i=n} (b_i e^{-a_i}) \quad (\textstyle\sum\text{: Summation Symbol})$$

(An alternative procedure involving integrated form of transmission $I_o \int e^{-a} db$ is described in the Appendix).

Considering the latter, more general case, the total intensity reaching the 'hole' becomes

$$(c - b)I_o + I_o \sum_{i=1}^{i=n} (b_i e^{-a_i})$$

and the photometer reading θ_{20}, proportional to the combined intensity, is

$$\theta_{20} = \chi\left\{(c-b)I_o + I_o\sum_{i=1}^{i=n}(b_i e^{-a_i})\right\} \tag{1}$$

where χ is the constant of proportionality.

The X40 objective: The X40 objective with its higher numerical aperture will gather more light and will also disperse more. If these factors are included in a common constant 'K_1', the intensity *per unit area* transmitted through the background, reaching the 'hole', becomes $K_1 I_o$ and that for the background surrounding the cell (Fig. 3) $(c-4b)K_1 I_o$. The intensity transmitted through the *nonhomogeneous* cell, reaching the 'hole' is now $4K_1 I_o \sum_{i=1}^{i=n}(b_i e^{-a_i})$; the areas ($b_n$) of the individual regions being magnified four times.

The photometer reading θ_{40} proportional to the sum of the two intensities is

$$\theta_{40} = \chi\left\{(c-4b)K_1 I_o + 4K_1 I_o \sum_{i=1}^{i=n}(b_i e^{-a_i})\right\} \tag{2}$$

It may be noted that the change over from X20 to X40 objective has not altered the characteristic of the cell to absorb light. The objective only spreads the transmitted intensity. As a result, the distribution of intensity between the image of the cell and the surrounding clear area, within the 'hole', is different with the two objectives and this is given by the equations (1) and (2).

The term cI_o gets eliminated after solving equations 1 and 2. Hence,

$$\left(\theta_{20} - \frac{\theta_{40}}{K_1}\right) = 3\chi I_o\left\{b - \sum_{i=1}^{i=n}(b_i e^{-a_i})\right\}$$
$$= 3\chi I_o\{b - b_1 e^{-a_1} - b_2 e^{-a_2} - \ldots - b_n e^{-a_n}\}$$

If we write $e^{-a_1} = 1 - a_1 + \frac{a_1^2}{2}\ldots$ and similarly for the other exponential terms and ignore the terms like $\frac{a_1^2}{2}$ and further, then

$$\left(\theta_{20} = \frac{\theta_{40}}{K_1}\right) = 3\chi I_o\{b - b_1(1-a_1) - b_2(1-a_2) - \ldots - b_n(1-a_n)\}$$
$$= 3\chi I_o\{b - (b_1 + b_2 + \ldots + b_n) + (a_1 b_1 + a_2 b_2 + \ldots + a_n b_n)\}$$
$$= 3\chi I_o\left\{b - b + \sum_{i=1}^{i=n}(a_i b_i)\right\}$$
$$= 3\chi I_o \sum_{i=1}^{i=n}(a_i b_i)$$

Hence $\sum_{i=1}^{i=n}(a_i b_i)$ = Total absorbance of the cell

$$= \frac{1}{3\mathrm{X}I_o}\left(\theta_{20} - \frac{\theta_{40}}{K_1}\right) \tag{3}$$

In arbitrary units, the intensity I_o is proportional to the background photometer reading (B_{20}) *per unit area*. As the voltage of the lamp remains the same for both objectives, I_o can be replaced by B_{20} or else omitted as it becomes a constant.

The constant of proportionality 'χ' can be determined by using sources of known candle power. However, in practice, 'χ' can be taken to be unity. The constant K_1 expresses relation between

transmissions through X40 and X20 objectives, for the same intensity of incident light, and is numerically equal to the ratio of photometer readings B_{40} and B_{20} for the background. With reference to the experimental condition of this work, K_1 equals the inverse ratio of magnifying powers of the two objectives. Hence,

$$K_1 = \frac{B_{40}}{B_{20}} = \frac{X20}{X40} = \frac{1}{2}$$

The cellular absorbance $(A)_{20/40}$ for the pair of objectives X20 and X40 is

$$(A)_{20/40} = \sum_{i=1}^{i=n} (a_i b_i) = \frac{1}{3B_{20}} (\theta_{20} - 2\theta_{40}) \tag{4}$$

with $x = 1$, $K_1 = 1/2$ and $I_o = B_{20}$

The equation (4) is independent of the contribution to intensity from the clear area in the 'hole' surrounding the cell. The background reading, B_{20}, can be dispensed off if the voltage of the lamp remains constant for all the observations in a given experiment.

Similar equations can be obtained with other pairs of objectives such as X20, X63 and X40, X63. Thus, *with reference to X20 objective*,

$$(A)_{40/63} = \sum_{i=1}^{i=n} (a_i b_i) = \frac{1}{5.92B_{20}} (2\theta_{40} - 3.15\theta_{63}) \tag{5}$$

with $x = 1$, $K_1 = 1/2$, $K_2 = 20/63 = 1/3.15$, $I_o = B_{20}$

$$(A)_{20/63} = \sum_{i=1}^{i=n} (a_i b_i) = \frac{1}{8.92B_{20}} (\theta_{20} - 3.15\theta_{63}) \tag{6}$$

with $x = 1$, $K_2 = 20/63 = 1/3.15$, $I_o = B_{20}$

The limitations of equations (4), (5) and (6) arising of omission of terms $a^2/2$ and further, in the exponential series e^{-a}, will be discussed later.

Results

The following photomicrographs (Figs. 2*A*, 2*B*, 6*A*, 6*B*), corresponding to different experimental conditions, were obtained using the experimental setup illustrated in Fig. 1.

For the ascites cells stained with Giemsa: X40 objective, 'hole' diameter 4.0 mm (Fig. 2*A*) and X20 objective, 'hole' diameter 4.0 mm (Fig. 2*B*).

For the Human Amnion (HA) *cells stained with Sudan Black* B (The cells were not pretreated before staining and hence the nuclei appear colourless): X40 objective, 'hole' diameter 2.5 mm (Fig. 6*A*, the 'hole' covers the nucleus of a cell). X40 objective, 'hole' diameter 2.5 mm (Fig. 6*B*, the 'hole' covers background adjacent to the cell).

The low values of numerical apertures of the three objectives (0.4 for X20, 0.65 for X40 and 0.9 for X63) ensure that the image focussed on the screen is

Table I. (Refer to Photomicrographs 2A, B)

Objectives	X20		X40		X63		Absorbance X 10^2		
Photometer reading	θ_{20}	B_{20}	θ_{40}	B_{40}	θ_{63}	B_{63}	$(A)_{20/40}$	$(A)_{40/63}$	$(A)_{20/63}$
							(Arbitrary Units)		
No. of Observation									
1	93.0	95.0	43.0	47.5	23.0	30.0	2.40	2.40	2.42
2	89.0	93.0	40.0	46.5	19.5	29.5	3.20	3.30	3.30
3	71.5	75.0	32.0	37.5	15.0	23.5	3.30	3.60	3.70
4	67.5	70.0	29.5	35.0	13.5	22.0	4.04	3.97	4.00
5	82.0	85.0	37.0	42.5	18.5	27.0	3.13	3.11	3.13
6	89.0	92.0	40.0	46.0	20.0	29.0	3.20	3.16	3.20
7	76.0	80.0	33.0	40.0	—	—	4.16		
8	74.0	80.0	31.0	40.0	—	—	5.00		
9	72.0	80.0	25.0	40.0	—	—	9.16		
10	69.0	80.0	23.0	40.0	—	—	9.57		

The photometer readings θ_{20}, θ_{40}, θ_{63} the corresponding background readings B_{20}, B_{40}, B_{63} and absorbance (A) are for the ascites cells stained with Giemsa. The numerical apertures of objectives X20, X40 and X63 are respectively 0.45, 0.65 and 0.9.

—: The readings θ_{63} were unmeasurably low for deeply stained cells 9 and 10.

also in focus at the level of the mirror. This was confirmed by taking two photographs (not presented) of the same field, at the same setting of focussing, one at the level of the screen an the other at the level of the mirror.

The following total absorbance values were obtained by repeated observations on a Giemsa stained ascites cell using the pair of objectives X20, X40, and a mirror with a 'hole' of diameter 4 mm: 3.16, 2.07, 3.25, 3.21, 3.19, 3.00, 3.17, 3.20, 3.02, 3.16. The standard error is ± 0.026.

The photometric readings (arbitrary units) for the ascites cells from the peritoneal cavity of Swiss mice are given in Table I. The cells were routinely stained according to the Giemsa method (Fig. 2*A*, *B*). The readings θ_{20}, θ_{40} and θ_{63} for each cell refer to the objectives X20, X40 and X63 respectively. Any two of these readings substituted in the appropriate mathematical equations (4), (5) and (6) gives total absorbance of the cell.

The pairs of objectives X20, X40; X20, X63 and X40, X63 thus give three independent values of total absorbance for the same cell and these agree with each other (Table I).

The performance of the 'two-objective, one-area' procedure, suggested in this work, was assessed by the most frequently measured object in this field — the stained nucleus of a cell. The following two experiments were carried out: a. Estimation of ploidy in liver cells of growing mice. b. Effect of hydroxyurea (HU) treatment on the synthesis of DNA in Human Amnion (HA) cells in tissue culture.

a. The livers of three one week old Swiss mice were removed, single cell suspensions made, washed thrice with balanced salt solution and suspended in saline. The suspension was suitably diluted to give smears on glass slides of fairly well distributed single cells. The cells were routinely fixed with neutral buffered Formalin, stained with Feulgen procedure and embedded with DPX mountant. The stained nuclei were scored for their DNA content, measured in terms of nuclear absorbance at $520 \pm 20\,\mu$m, using the 'two-objective, one area' procedure.

b. The HA cells in tissue culture were grown on glass coverslips (5×10^5 cells/ml of F-10 medium) in Leighton tubes for 48 hours. The cells were then treated with HU (Fluka, Mol. Wt. 76.06), 0.76 mg/ml of medium, for 12, 24 and 48 h. Appropriate controls for each treatment were kept in plain medium. The cells were fixed with neutral buffered Formalin, stained routinely with Feulgen procedure and embedded with DPX mountant. The fixation and staining was identical for all the coverslips. The cells were scored at $520 \pm 20\,\mu$m, for DNA, using the 'two-objective, one-area' procedure.

Discussion

The procedure outlined in the 'two-objective, one-area' method shows how the photometric absorption measurements of stained object with two different objectives enables determination of absorbance of the object.

The total absorbance ($\sum_{i=1}^{i=n} a_i b_i$) in equations (4), (5), (6) is obtained after ignoring the terms such as $a^2/2$ and further in the exponential series e^{-a}. For a stained cell having absorbance *per unit area* 'a' $= 0.3$, $e^{-a} = e^{-0.3} = 0.7415$ and $1 - a = 0.7$. Hence, the error in assessing $e^{-0.3} = (1 - 0.3)$ is $\frac{0.0415}{0.7415} \times 100 =$ $= 5.6\,\%$ and rises rapidly above this.

Further, the term absorbance (a) and transmittance (T) are related by the equation:

$$a = -\log_{10} T = \frac{\textit{Intensity transmitted through the cell}}{\textit{Intensity incident on the cell}}$$

Hence, with $a = 0.3$, T^{-1} is about 2.0 and intensity incident on the cell $= 2.0$ X intensity transmitted through the cell.

Thus, for a cell or its part, absorbing about 50 % of incident light, the absorbance *per unit area* is 0.30 and the error in evaluating its total absorbance by the 'two-objective, one-area' method is 5.6 %.

From Table I, for a cell showing the highest total absorbance of 9.57×10^{-2} (arbitrary units), the photometric readings θ_{20} and θ_{40} are respectively 15 % and 42.5 % off the corresponding background readings B_{20} and B_{40}. Considering that the clear area in the 'hole', surrounding the cell, also contributes to the readings θ_{20} and θ_{40}, it can be said that the absorption of intensity by the above cell is less than 50 %.

In general, equations (4), (5) and (6) are suitable for fairly deeply stained cells for which the photometric readings 'θ' are within 50 % of the corresponding readings 'B' for the background and under this condition the error in measuring total absorbance is around 5.6 %.

The subject of errors in absorption microphotometry has been thoroughly discussed (Garcia 1962; Mendelsohn 1966; Swift and Rasch 1956). The distribution error can be kept within 3 % only by the scanning method (Caspersson and Lomakka 1972). The error due to contribution to intensity from the clear area in the measuring aperture, surrounding a stained specimen, can be minimized up to 10 % by employing 'two-wavelength, one-area' (Garcia 1962) or 'Two-area, one-wavelength' (Garcia and Iorio 1966) methods and is eliminated in the 'two-objective, one-area' method of this work.

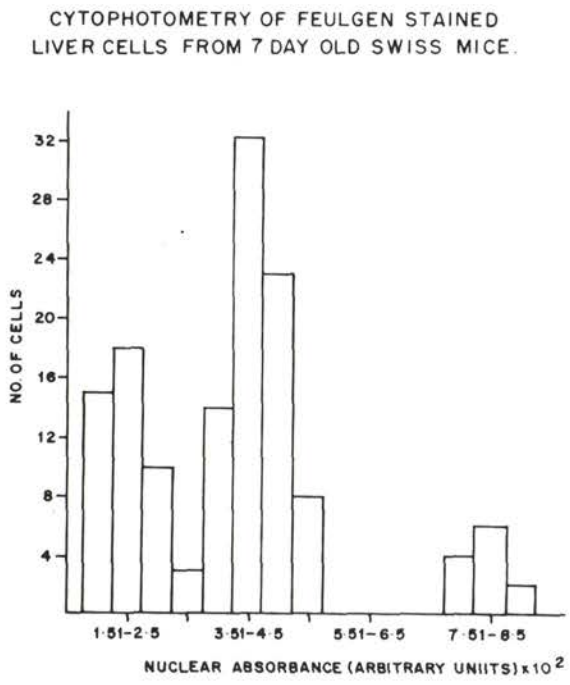

Fig. 4. Histogram showing distribution of DNA, measured as nuclear absorbance at $520 \pm 20\,\mu m$ in the nuclei of liver cells of growing Swiss mice. The diameter of the measuring 'hole' was 4 mm.

The criterion of accuracy in absorption microphotometry is generally set at error not exceeding 10 %. Proceeding as above, for $a = 0.4$, $e^{-a} = e^{-0.4} = = 0.6701$ and $1 - a = 0.6$. Hence, the error in assessing $e^{-0.4} = (1 - 0.4)$ is $\frac{0.0701}{0.6701} \times 100 = 10.45\,\%$. The 'two-objective, one-area' method could therefore be considered suitable for specimens stained to produce absorbance *per unit area* of 0.4.

A pragmatic application of equations (4), (5) and (6) is seen from the measurements on ascites cells stained with Giemsa (Table I). These cells (Figs. 2*A*, *B*) appear nearly circular and only cells whose magnified image falls within the area of the 'hole' were scored. From the readings for the background intensity (B_{20}, B_{40}, B_{63}) it is clear that their ratio, for a given pair of objectives, equals inverse ratio of respective magnifying powers.

The readings 2 and 3 (Table I) are related to the same cell, but under different lamp voltages. The readings 5 and 6 are likewise noted for a different cell. These observations show that whatever the intensity of the lamp, as long

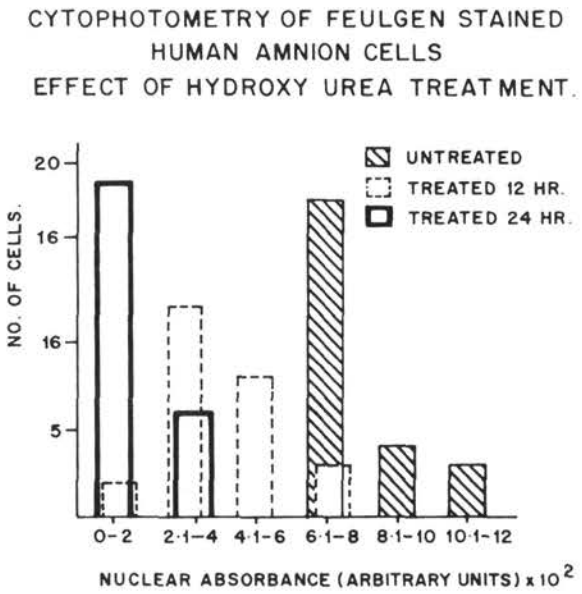

Fig. 5. Histogram showing distribution of DNA, measured as nuclear absorbance at 520 ± 20 μm, in the nucleous of Human Amnion cells in control and after treatment with hydroxyurea for 12 and 24 h. The diameter of the measuring 'hole' was 4 mm.

as it remains constant, during observations with a pair of objectives, the total cellular absorbance is almost the same.

The histograms of nuclear absorbance in arbitrary units against the cell number, in Fig. 4, show that the cells could be grouped around peak values in the range 1.5 to 2.5, 3.5 to 4.5 and 7.5 to 8.5. It thus shows, in agreement with Grundmann (1967), the occurrence of polyploidy in liver cells of growing mice.

Similar histograms of Fig. 5 show that the nuclear absorbance is highest for the cells in control and is progressively less for the cells treated with hydroxyurea (HU) for 12 and 24h. The 48h treatment gave unmeasurably low values of nuclear absorbance. The inhibition of DNA synthesis, thus obtained in cells treated with HU, is an agreement with the earlier report (Yarbrow et al. 1965).

The standard error from repeated observations on an ascites cell (see Results), using a pair of objectives X20, X40; is ± 0.026 i. e. 1 % and compares well with the standard of precision required in absorption microphotometry.

The error arising of light glare on the lens surface and lens barrel, monochromaticity of light, constancy of lamp voltage, fixation and staining of biological objects, scattering of light, are common in such work and could be minimized with the presently available techniques (Sandritter 1966).

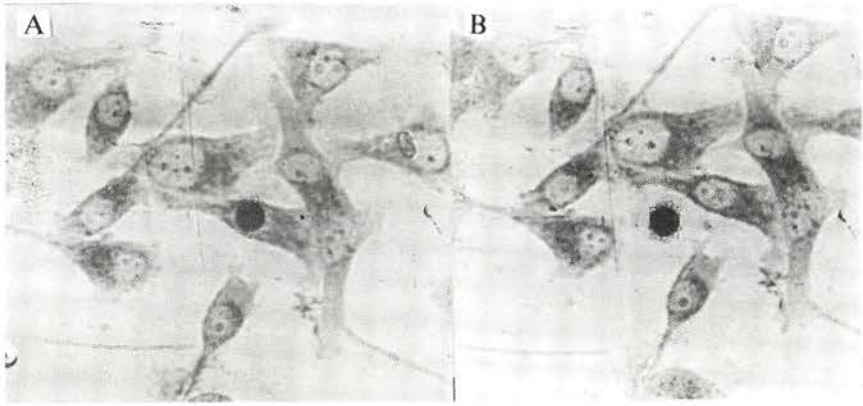

Fig. 6. Photomicrographs of Human Amnion cells, stained with Sudan Black B (E. Merck, Germany), under various conditions. The dark spot corresponds to the 'hole' in the mirror (Fig. 1). *A*: The image of the nucleus of a cell with X40 objective, brought over the 'hole' of diameter 2.5 mm. *B*: The 'hole' covering background adjacent to the cell in A.

The microphotometer assembly shown in Fig. 1 could also be operated with a single objective. In this procedure, the transmitted intensities are measured when the selected region (nucleus, Fig. 6*A*) and the adjacent background (Fig. 6*B*) are successively moved over the 'hole'. The ratio of the two intensities would give transmittance (T) provided the magnified cellular region (nucleus) snugly fits into the measuring 'hole'. It can be accomplished, nearly or wholly, by selecting an objective with appropriate magnifying power or a mirror with appropriate size of the 'hole'. However, the accuracy of such measurements will be limited by the distribution error. Yet the procedure of constructing a photometric device, using a reflecting mirror with a 'hole' is simple and precise and besides it permits measurement of transmission in a part of a cell while the rest of the cell remains in the field of view.

The limitation of the 'two-objective, one-area' method should be seen with its possible pragmatic application using the presently available computerised and programmable technology, coupled to highly accurate digital meters, and not with the manually operated model deviced in this work.

Appendix

The term $I_o \sum_{i=1}^{i=n} (b_i e^{-a_i})$ of equation '1' could be replaced by its integrated form $\int I_o e^{-a} db$ where the integration is carried out over the entire surface area 'b' of

the cell and it is assumed that the absorbance 'a' per unit area varies from point to point on the cell surface. Hence,

$$\theta_{20} = \chi\{(c - b)I_o + I_o\int e^{-a}db\} \quad (7)$$

$$\text{and } \theta_{40} = \chi\{(c - 4b)K_1I_o + 4K_1I_o\int e^{-a}db\} \quad (8)$$

Eliminating CI_o from these equations and writing $e^{-a} = (1 - a)$, after neglecting terms $a^2/2$ and further, we get

$$\int a\,db = \text{Total absorbance of the cell} = \frac{1}{3B_{20}}(\theta_{20} = 2\theta_{40})$$

with $x = 1$, $K_1 = 1/2$, $I_o = B_{20}$. This is the same as equation (4) derived earlier.

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References

Caspersson T., Lomakka G. (1972): Scanning microscopy technique for high resolution quantitative cytochemistry. Ann. N. Y. Acad. **97**, 449—463

Garcia A. M. (1962): Studies on DNA in leucocytes and related cells of mammals II. On the Feulgen reaction and two-wavelength microspectrophotometry. Histochemie **3**, 178—194

Garcia A. M., Iorio R. (1966): A one wavelength, two area method in cytophotometry for cells in smears of prints. In: Introduction to Quantitative Cytochemistry. (Ed. G. L. Weid), pp. 239—245, Academic Press, New York and London

Grundmann E. (1967): Studies with autoradiography and cytochemistry in the rat liver following partial hepatectomy. In: Control on Cellular Growth in Adult Organisms. (Eds. H. Teir and T. Rytomas), pp. 250—259, Academic Press, New York and London

Mendelsohn M. L. (1966): Absorption cytophotomery: Comparative methodology for heterogenous objects to quantitative cytochemistry. In: Introduction to Quantitative Cytochemistry. (Ed. G. L. Weid), pp. 201—214, Academic Press, New York and London

Pearse A. G. V. (1972): Histochemistry — Theoretical and Applied. Vol. 2, pp. 1225—1259, Churchill Livingstone, Edinburgh and London

Sandritter W. (1966): Methods and results in quantitative cytochemistry. In: Introduction to Quantitative Cytochemistry (Ed. G. L. Weid), pp. 159—182, Academic Press, New York and London

Swift H. H., Rasch E. (1956): Microphotometry with visible light. In: Physical Technique in Biological Research (Eds. G. Oster and A. W. Pollister), pp. 353—400, Academic Press, New York

Yarbrow J. W., Kennedy B. J., Barnum C. P. (1965): Hydroxyurea inhibition of DNA synthesis in ascites tumor. Proc. Nat.Acad. Sci. U.S.A. **53**, 1033—1035

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