Biochemical Pharmacology, 1960, Vol. 4, pp. 104–112. Pergamon Press Ltd., Printed in Great Britain.

SCANNING APPARATUS FOR THE QUANTITATIVE ESTIMATION OF DEOXYRIBONUCLEIC ACID CONTENT

E. M. DEELEY

King's College, University of London

Résumé—Les méthodes conventionnelles (*aperture methods*) et les méthodes par *scanning* pour l'estimation rapide et précise de l'absorption totale de matériel biologique sont brièvement passées en revue et une méthode de *scanning*, qui a été utilisée pour des estimations après réaction de Feulgen, est décrite plus en détails. Les limitations physiques et les avantages relatifs de la *scanning method* et de l'*aperture method* à deux longueurs d'onde dans différentes applications sont rapidement discutées.

INTRODUCTION

THE photometric methods available for the quantitative estimation of deoxyribonucleic acid (DNA) and other substances in biological material may be divided broadly into two groups:

(1) Aperture methods, in which the light from the whole of the measured field, defined by an aperture, falls on the measuring instrument at once.

(2) Scanning methods, in which the light from small areas of the measured field falls on the measuring instrument at successive intervals in time.

The aperture method in its simplest form affords the simplest method of measuring the total amount of absorbing material within a given area. It is well known, however, that this measurement depends to some extent on the way in which the absorbing material is distributed over this area (see for example DAVIES and WALKER, 1953). If, therefore, it is assumed that the material is evenly distributed over the measured area, there will be an error in the measurement which is generally known as the "distributional error". This error is small if the maximum optical extinction is small (e.g. it is not greater than 5 per cent if the extinction is never greater than 0·1 units), but increases rapidly with increasing extinction.

Under certain circumstances, however, it is possible to make use of a much improved aperture method known as the "two-wavelength" method (PATAU, 1952), in which the distributional error is largely corrected. This method makes use of the variation of optical extinction with wavelength (i.e. the absorption curve) of the material to be estimated, and is described in principle below.

Suppose a single measurement of optical extinction is first made at a given point on the specimen and at a certain wavelength (corresponding, say, to the absorption maximum) giving a value e_1. A further measurement at the same wavelength is then made of the average optical extinction E_1 over the whole area of the specimen. (This will inevitably include a certain area of the field surrounding the specimen.) These two measurements are then repeated at a different wavelength,

giving a smaller value of extinction e_2, and of average extinction E_2. Since the distributional error increases as the optical extinction increases, it follows that:

$$\frac{E_2}{e_2} \geqslant \frac{E_1}{e_1}$$

Furthermore, it can be seen that these measurements give some information on the way in which the material is distributed. For instance, if the two sides of the above equation were equal, it would follow that the material was evenly distributed, and there would be no distributional error. It has been shown (PATAU, 1952) that the inequality in the above equation affords a simple method of calculating approximately the distributional error, which can then be used to correct the measurement of average extinction E_1.

It is seen that this method requires that the absorption curves of all absorbing substances in the field are of the same shape or, as is usually the case, that only one absorbing substance is present.

In the scanning method distributional error is minimized by measuring the absorption of only a very small area of the specimen at one time, and then adding the results of successive measurements. Distributional error may be regarded as arising from the fact that the relationship between optical extinction (which is proportional to the amount of material present) and transmitted light is not linear but logarithmic. This is stated in the Beer–Lambert law, which may be written in the form:

$$\log \frac{I_0}{I_t} = k\left(\frac{dM}{dA}\right)$$

Where I_0 and I_t are the intensities of the incident and transmitted light, dM/dA is the surface concentration of material (or mass/unit area) and k is a constant.

Integrating over the whole area of the field:

$$M = \int_A [(1/k) \log I_0 - (1/k) \log I_t] dA$$

It is this integral which is, in effect, evaluated in the scanning method. An apparatus which has been designed (DEELEY, 1955) to perform this operation will now be described.

THE INTEGRATING MICRODENSITOMETER

Principle of operation

A block diagram of the apparatus is shown in Fig. 1. A small moving aperture (at the scanning disk) is provided in the image plane of a microscope focused on the specimen being measured. Behind this moving aperture is mounted a photomultiplier.

If light of intensity I is permitted to pass through an aperture of area δA on to the photomultiplier operated at constant voltage, the current generated at the anode will be equal to $KI\delta A$, where K is a constant depending on the sensitivity of the photocathode which will vary according to the position of δA in the field. If the

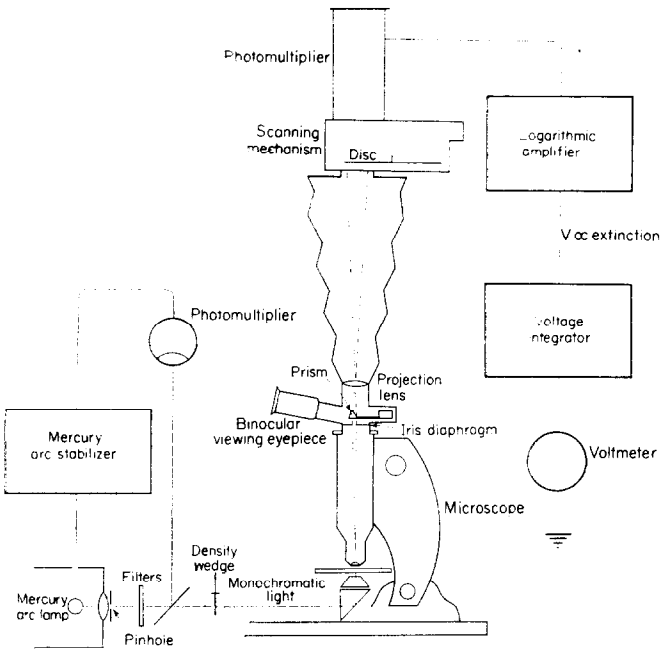

FIG. 1. The integrating microdensitometer—schematic diagram.

currents actually generated, with the specimen in position and out, are i_t and i_0, the above equation may be rewritten:

$$M = \int_A [(1/k) \log (i_0/K\delta A) - (1/k) \log (i_t/K\delta A)]dA$$

or

$$M = (1/k) \int_A \log i_0 \, dA - (1/k) \int_A \log i_t \, dA$$

Thus K and δA disappear from the equation, which means that as the aperture scans the field any variations in these quantities will have no effect on the calculation of M.

In practice the photomultiplier is not operated at constant voltage, but in a circuit which gives a voltage output directly proportional to $\log i$. A voltage integrating circuit then gives a signal V proportional to $\int -\log i \, dt$. If, therefore, the scanning spot is made to move at a constant rate, so that dA/dt is constant, M will be proportional to $V_t - V_0$, the difference between the output voltages of the integrator with and without the specimen in position.

The scanning mechanism

The scanning aperture is formed by the intersection of two narrow slits which are approximately at right angles to one another. The scanned area A (Fig. 2) is about 1·5 cm in diameter. Twenty radial slots 0·2 mm in width and about 8 mm in length are cut around the circumference of a disk, 10 cm in diameter. This disk is driven by a synchronous motor rotating at 1 rev/sec. A further single slot is cut in a plate K at right angles to the radius of the disk, so that as the disk rotates an aperture

formed at the intersection of the slits moves across the area *A*. The motor, disk and plate *K* are free to move in the direction indicated by the arrow (Fig. 2) and are driven to and fro by the action of a cam which rotates once every 3 sec. In this way the area *A* is scanned by a succession of apertures once every 3 sec. In practice a microswitch stops the motor at the end of each cycle of the cam, so that each scan is under the control of the operator. Owing to the angle between slits, the aperture moves slightly faster at the edges of the area *A* than in the centre. This non-uniformity introduces a maximum error of 0·5 per cent at the edges of the field.

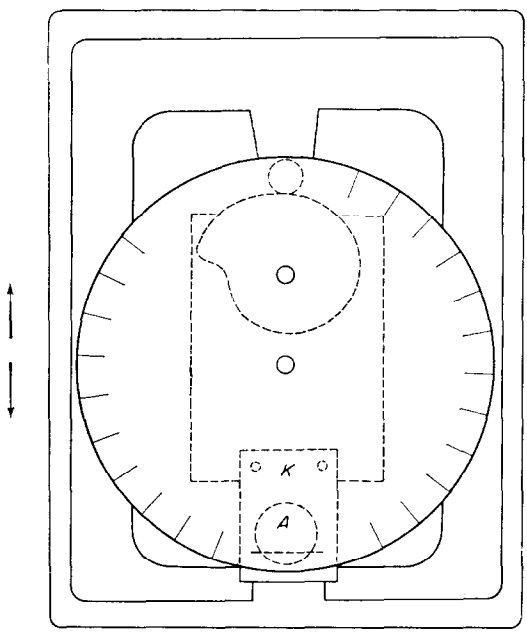

FIG. 2. The scanning mechanism.

The optical system

Estimations of Feulgen stain can be conveniently made at 546 mμ, the green line of the mercury spectrum. This leads to a simple optical system whereby the specimen is illuminated according to KÖHLER from a 125 W mercury vapour lamp using the appropriate light filters. In practice pin-hole stops are used at the field lens to minimize the effect of glare. A circular photographic density wedge is also included in the light beam to regulate the light level in the microscope. An electrically-operated prism enables the image of the specimen which is formed at the iris diaphragm to be examined by a viewing eyepiece of the binocular type. When the prism is removed from the light path a projection lens forms a magnified image of the iris diaphragm at the scanning disk. Leather bellows prevent stray light from entering the system.

For most specimens a specially-designed microscope condenser is used whereby the specimen to be measured is crushed, redistributing the material into one plane and reducing the optical extinction (DAVIES *et al.*, 1954). Errors arising from parts

of the object lying outside the depth of focus of the microscope, from unwanted stray light, and from the apparent increase in extinction due to some rays from the condenser passing obliquely through the object are thereby reduced to a minimum.

Stabilization of light intensity

Fluctuations in light intensity (caused mainly by the movement of the arc of the mercury vapour lamp) may be as much as 10 per cent of the total light intensity reaching the microscope, so that some form of stabilization is essential. A small fraction (about 4 per cent) of the light from the field lens falls on to a second photomultiplier as shown (Fig. 1). The anode current of this photomultiplier is held constant by a feedback circuit which controls the current in the arc lamp. In this way variations in light intensity are reduced to a fraction of 1 per cent.

The integrator circuit

The integrator circuit is of the "Miller" type, using a single-stage d.c. amplifier with resistive input and capacitative feedback. The integrator is preceeded by a diode switch which connects the output of the logarithm-law photomultiplier circuit to the integrator only when the optical extinction measured by the circuit has fallen below a certain prescribed value. The integrator is thus prevented from recording a very large extinction when the scanning aperture moves outside the illuminated field. The integrator output is displayed on a voltmeter.

The densitometer is controlled by two microswitches, one to start the scanning motor, switch on the integrating circuit and remove the viewing eyepiece prism from the light path, and the other to cancel the integrator reading at the end of each 3 sec cycle and to re-introduce the prism.

Performance

Without moving the specimen, small variations in successive readings of the integrator output voltage occur owing to photomultiplier fluctuations and small irregularities in the operation of the scanning mechanism. The random error thereby introduced into the measurement of stain content is largest for small,

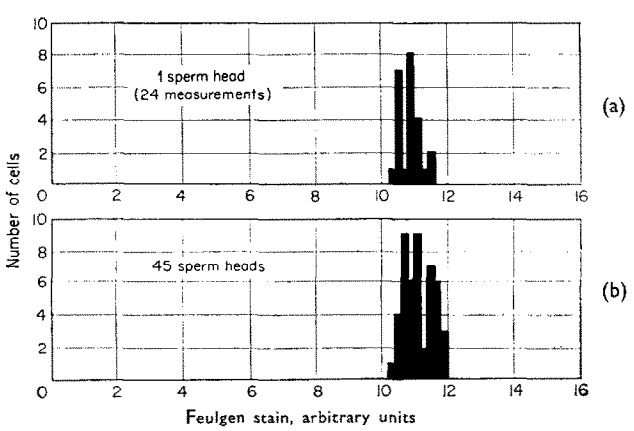

FIG. 3. Measurements on ram sperm heads.

lightly-stained objects (e.g. ram sperm heads). In practice, the effect of these variations is reduced by taking the mean of three successive readings of each object and subtracting the mean of three readings of clear field. Fig. 3(a) illustrates the results of twenty-four independent determinations of the Feulgen stain in a single ram sperm head, for which the mean error is $\pm 1{\cdot}5$ per cent.

In Fig. 3(b) is illustrated the results of measurements on forty-five different sperm heads, from which it can be seen that there is little significant difference between the distributions, and therefore there can be little real variation in the stain content of the measured cells.

Use has been made of the above result to verify freedom from distributional error in the present apparatus. The average amount of stain in one sperm head as calculated from measurements on thirty specimens has been compared with the amount of stain in each of several groups of two or more sperm heads, in which the specimens overlap to varying extents. It has been found that in each group the amount of stain corresponds to the correct multiple of the average stain in each specimen to within the expected variation due to the material, so that no significant distributional error is present.

A second method of verifying freedom from distributional error makes use of the crushing technique (DAVIES *et al.*, 1954) which has been used to redistribute in one plane the material of Feulgen stained cells of onion-root tip. When nuclei of various forms are crushed until the whole material lies within the depth of focus of the microscope, it is found that the measured amount of stain is independent of further crushing, to within the random error of the instrument.

The effect of defocusing and glare have also been examined (DAVIES, 1955) and in normal circumstances have a negligible effect on the accuracy of the instrument.

A COMPARISON OF METHODS FOR TOTAL ABSORPTION MEASUREMENTS

Physical considerations

The apparatus used in scanning methods is somewhat more complex than that required for the two-wavelength method. In the latter method the electrical measuring circuits are relatively simple, the main complication being the provision of an adjustable monochromator. In a scanning system there is considerably greater complication in the mechanical components and electrical measuring circuits, and only for certain applications (e.g. measurements on Feulgen stain at 546 mμ) is the optical system less elaborate.

The physical disadvantage of the scanning technique is that light from each element of area reaches the measuring device for only a small fraction of the total measuring time. It follows that the error due to random variations in or after the measuring device will be correspondingly greater than when using an aperture method. A scanning technique therefore requires a more intense light source or a longer total measuring time.

For measurements in visible light, however, the scanning method has been found to be more convenient, and in many applications more rapid to use, while in ultra-violet light it is usually the only practicable method. It is therefore appropriate briefly to compare the two methods in different applications.

Estimations at 546 mμ

For estimations of Feulgen or other stains at 546 mμ (the green line of the mercury spectrum) there is adequate light to scan the image of most cell nuclei in 3 sec with negligible random error. Bearing in mind the time taken to move and select a specimen, to adjust apertures, etc., this time is about the minimum that can be usefully employed, and the scanning method has been found to be far more rapid and convenient than the two-wavelength method in this application.

Absorption measurements by the scanning and two-wavelength methods have been compared (MENDELSOHN and RICHARDS, 1958) using nuclei of an Ehrlich ascites tumour stained with gallocyanin-chrome alum. As shown in Fig. 4, there is

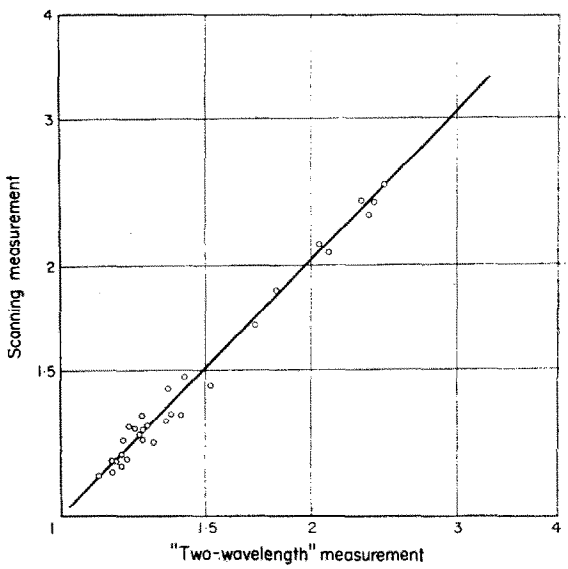

FIG. 4. Comparison of methods (from MENDELSOHN and RICHARDS).

close agreement between the two methods for the stain content of the thirty nuclei measured, which included several nuclei in various stages of division.

Other estimations in visible light

For estimations at wavelengths different from that of the green line of the mercury spectrum the amount of light available at a comparable bandwidth is not so great, and it would then be necessary to increase the total measuring time from 3 sec. Frequently, however, the maximum of the absorption spectrum of the substance to be estimated is not sharp, so that a wider bandwidth can be tolerated. Thus a high-power source giving a continuous spectrum (e.g. the high-pressure xenon arc lamp) used in conjunction with a monochromator, or with interference filters if the bandwidth is not critical, can in some circumstances give sufficient light to maintain a scanning rate of 3 sec. If the bandwidth must remain narrow, or if measurements are made near the red end of the spectrum, a longer scanning time would be required so that in these circumstances the method would lose some advantages over the two-wavelength method.

Estimations in ultra-violet light

In making use of the natural ultra-violet absorption of cellular constituents difficulties arise due to the multiplicity of absorbing substances, and to light scatter at shorter wavelengths. Furthermore there is generally no clear, non-absorbing field around the particular area to be measured, and that area may in itself include areas which we might wish to exclude from the measurement. In these circumstances aperture methods are at a serious disadvantage and scanning techniques offer the only practicable approach. It is possible, using a scanning technique, to devise electrical or mechanical means of defining specific areas of the field over which the measurement is to be made, while excluding other areas. This facility is almost impossible to achieve using aperture methods.

SUMMARY

The "aperture" and "scanning" methods for the rapid and accurate estimation of the total absorption of biological cells is briefly reviewed, and a scanning method which has been used for Feulgen estimations described in more detail. The physical limitations and relative advantages of the scanning method and the "two-wavelength" aperture method in various applications is briefly discussed.

REFERENCES

DAVIES H. G. and WALKER P. M. B. (1953) *Progress in Biophysics* (Edited by BUTLER, J. A. V. and RANDALL J. T.) Vol 3, p. 195. Pergamon Press, London.

DAVIES H. G., WILKINS M. H. F. and BODDY R. G. H. B. (1954) *Exp. Cell Res.* **6**, 550.

DEELEY E. M. (1955) *J. Sci. Instrum.* **32**, 263.

MENDELSOHN M. L. and RICHARDS B. R. (1958) *J. Biophys. Biochem. Cytol.* **4**, 707.

PATAU K. (1952) *Chromosoma* **5**, 341.

DISCUSSION

J. FAUTREZ: J'ai été très intéressé par la comparaison des résultats obtenus par la *scanning method* et l'*aperture method* en deux longueurs d'onde. Avec GOVAERT et ROELS, j'ai pour ma part comparé dans des noyaux d'origine animale en interphase la méthode en deux longueurs d'onde et celle à une longueur d'onde. Les résultats sont absolument comparables. Je crois que pour l'étude de la teneur en ADN des noyaux interphasiques la méthode à une longueur d'onde donne d'aussi bons résultats que celle à deux longueurs d'onde et que le *scanning*; je la préfère dès lors parce que la plus simple.

Je peux montrer deux photographies: d'une part des spermatogonies de rats, d'autre part des vésicules germinatives des oocytes I. Dans les deux matériels, les conditions sont telles que l'on peut s'attendre à voir jouer au maximum des causes d'erreur telles que la *distributional error*. Les moyennes de cinquante mesures (Dr. VAN DE KERCKHOVE) donnent dans les deux cas la même valeur moyenne, à moins de 2% près.

E. M. DEELEY: It is true that using interphase nuclei and other nuclei in which the stained material is approximately evenly distributed, the error in a single-wavelength aperture measurement (as used by LISON, and by POLLISTER) may not be large.

J. DANIELLI: You suggest that a scanning machine can be programmed, e.g. to give nuclear content less nucleolus content. Are there not serious difficulties in this with cells of average size? CASPERSSON has calculated that to estimate accurately the content of a spherical body, it must have a diameter of 3λ. Such a condition presumably obtains with nucleoli.

E. M. DEELEY: It is probable that the elimination of the contribution of the nucleolus could not be achieved to a very great accuracy, but since this contribution is not usually a large proportion of the total nuclear absorption it may prove to be a useful technique in further reducing the error from this source in some material.

W. PLAUT: Have you any information on the use of your instrument with a more intense light source such as the xenon arc plus a monochromator?

E. M. DEELEY: We have not definite information on this. Toward the blue end of the spectrum it might be possible to operate the instrument simply by modifying the light-stabilizing circuit to meet the requirements of the new lamp. Toward the red end of the spectrum, however, other electrical mechanical modifications would be necessary.

T. CASPERSSON: After a question from the audience about light sources: In a high-speed scanning ultramicrospectrograph for ultra-violet and visible we have attained scanning times of the order mentioned also in u.v. by aid of super high-pressure mercury arcs and xenon arcs plus high quality optics.

E. M. DEELEY: Our experience with a 250 W xenon source was that a longer scanning time was needed in the ultra-violet, although we did not attempt to reduce this to the absolute minimum.

F. KASTEN:

(1) How long does it ordinarily take to measure forty-five different sperm heads?

(2) What will be the cost of the commercial instrument?

E. M. DEELEY: Using a good preparation of sperm heads (i.e. one in which the specimens are not too close together) it is possible to measure forty-five sperm heads in from 30 to 40 min. With less-conveniently selected nuclei a longer time is necessary. I believe that the present price is about $7000.