AN ACCURATE REFERENCE SYSTEM FOR HISTORADIOGRAPHY*

by

J. J. CLEMMONS AND T. C. WEBSTER

Department of Pathology, University of Wisconsin Medical School, Madison 6, Wisconsin (U.S.A.)

ENGSTROM[1] presented a theoretical discussion whereby the application of the X-ray absorption laws to historadiographs made it possible to determine the mass of small biological structures. The basis of this determination is the absorption of X-rays by a section of tissue and a reference system of approximately the same carbon, nitrogen, and oxygen composition as the average protein.

The reference system and section of tissue are simultaneously exposed to X-rays (8–12 A) and the image recorded on the high resolving Lippman emulsion. The use of a photo emulsion necessitates the introduction of a reference system in the form of a step wedge to determine the characteristics of the HURTER AND DRIFFIELD curve for the emulsion. The standards or nitrocellulose layers in the step wedge must be uniform and of known thickness to provide a range of densities comparable with that of the historadiograph. The range of thickness generally required for this is 0.2 to 5.0 μ. A complete wedge has a minimum of three steps.

Previous methods for preparing the reference system consisted of the wire loop technique for preparation of the foils and gravimetric or interferometric methods to determine the thickness[2,3]. A wire loop is plunged through the water surface in a recrystallization dish and a drop of the film forming solution is spread over the surface. When the film is dry the loop is raised into contact with the underside of the film, which can now be lifted. After the film dries and shrinks an area is cut out and weighed. From the known weight, area and density the thickness is calculated. Smaller squares are then cut and mounted with fine forceps on the supporting foil of the metallic disc. The procedure is quite tedious, and the pronounced number of interference patterns indicate non-uniformity. It is difficult to avoid wrinkles, and on checking evenness, it was found that there were 20–100% variations in the thickness of a foil prepared by the loop method. Interferometry provides an accurate method for determining thickness of these foils but requires special apparatus and is not readily applicable to the foils when arranged in the form of a wedge on a metal disc[4,5]. Gravimetric methods are valid only if the area weighed is perfectly even. This method is also tedious and cannot be used for the same foils arranged in the form of a wedge.

In the method to be described here, light absorption of colored "Parlodion" is used to calculate the thickness of the thin foils. The dye should have the following properties: (a) insoluble in water, but soluble in "Parlodion" and acetate, (b) possess good light

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absorption properties, (c) contain no elements of high atomic number or require such concentrations as to alter the absorption coefficient of the reference material, (d) must be stable to light. Drug and Cosmetic Green #6 (alizarin green) was found to meet these requirements. If the Lambert-Beer Law can be applied to the system, the intensity of the emerging light is in exponential proportion to the thickness of the layer and the concentration, the density d is a simple linear function of these quantities. d is directly proportional to the concentration c and the thickness l of the layer:

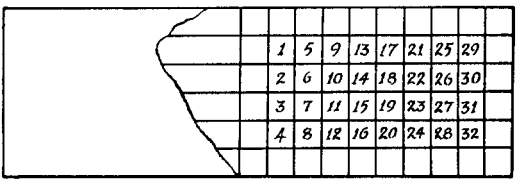

Fig. 1. Glass slides are dipped into a Parlodion-dye solution, dried and the surface cut.

$$\frac{d_1}{d_2} = \frac{c_1 l_1}{c_2 l_2} \qquad d = -\log T \tag{1}$$

When the concentration is fixed a variation in d is in direct proportion to any change in the thickness l.

Procedure. Two grams of "Parlodion" are accurately weighed and dissolved in 10 ml of ether + 10 ml of ethanol. Amyl acetate is saturated with D and C green #6 and then diluted (7 parts saturated solution + 3 parts of solvent). This solution is used to dilute the "Parlodion", alcohol, ether mixture to 100 ml. It is imperative that the solutions be filtered through a Seitz filter to remove microscopic particles. With a 635 mμ filter and a 1 cm l_1 cell, the density d_1, of a 1:50 dilution of this solution is determined. Clean glass slides are dipped into the undiluted solution and allowed to stand on end and dry. Once dry, the process is repeated depending on the thickness desired or range to be covered by the wedge. The surface of the dried parlodion is cut (Fig. 1), and the foils floated free from the glass slide by immersing it in water (Fig. 2). All foils except those marked are discarded.

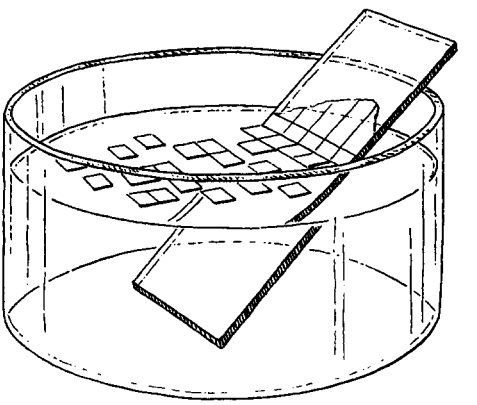

Fig. 2. The cut foils are stripped from the glass slide by immersing it into water.

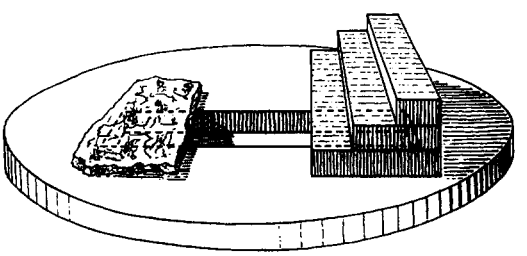

Fig. 3. Foils are mounted on metal disc. Illustration is not in proportion as the thickest foil is generally 0.1 to 0.5 the thickness of the tissue.

In quantitative historadiography a metallic disc with slit covered by a supporting parlodion foil is used to support both the tissue and reference wedge. The wedge is mounted over one-half of the slit in the metallic disc (Fig. 3). This is accomplished by placing the disc under a small foil that has been stripped from a glass slide. The foil is mounted on the disc as it is lifted from the water. It is dried (35° C) on the disc, and the process repeated

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until a wedge having the desired number of steps is formed. The foils are very uniform, and the reference wedge quickly constructed.

A microcolorimeter is used to determine the absorption d_2 for each step of the wedge and the thickness l_2 of the steps is calculated.

$$l_2 = \frac{d_2}{d_1} \cdot \frac{c_1}{c_2} \cdot l_1 \tag{2}$$

A factor is introduced to correct for differences in the absorption characteristics and changes that occur as the solvent evaporates leaving the dye dispersed in the "Parlodion".

$$K = \frac{d_1}{d_2} \cdot \frac{c_2}{c_1} \cdot \frac{\text{wt. foil}}{\text{A} \cdot \varrho\text{ref.}} \cdot \frac{1}{l_1} \tag{3}$$

A = area of foil weighed.

Uniform foils were prepared and from a gravimetric determination of the weight and a measurement of d_1 and d_2, K was calculated using equation (3). K for several solutions was found to have the value 1.35.

$$l_2 = \frac{d_2}{d_1} \cdot \frac{c_1}{c_2} \cdot l_1 \cdot K \tag{4}$$

If a 2% dyed "Parlodion" solution, diluted 1:50, is used and $l_1 = 1$ cm.

$$\frac{c_1}{c_2} = \frac{0.0004}{1.60}$$

$$l_2(\text{microns}) = \frac{d_2}{d_1} \cdot 2.5 \cdot 1.35 \tag{5}$$

Equation (4) is used to calculate the thickness of the reference system. The 635 mμ filter must have the same half-band width characteristics for the d_1 and d_2 values, but a correction factor may be introduced if it is not practical to use the same instrument for the d_1 and d_2 determinations.

As the d_1 value is for the parlodion + dye + acetate and the d_2 value for the dye in solution in parlodion, the spectral absorption curve for each condition was determined. There is no significant shift of the absorption curve (Fig. 4). Confirmation of the Lambert-Beer Law (0.2–5 μ) was made by determining the density of several foils stripped from a glass slide. The densities of the wedge constructed from these foils were compared to values obtained by adding known densities of the individual foils (Table I).

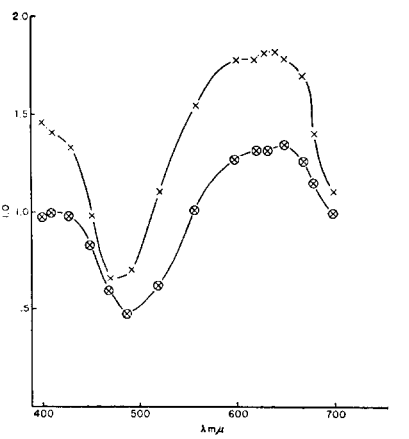

Fig. 4. × – absorption curve for the Parlodion-dye-acetate solution (d_1). ⊗ – absorption curve for the Parlodion-dye foil (d_2).

To determine the accuracy of the method glass slides were dipped and dried as previously described. Squares were cut on the surface and accurately measured with a microcomparator. The optical density was measured at many points over the entire area

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which gave the d_2 value and checked the area for uniformity. The thickness was calculated, and from this and the area the weight was determined. These values were compared with a gravimetric determination of weight of the same foils. The results are given in Table II.

TABLE I

	Foil d	*Theoretical wedge*	*Experimental wedge*
1	0.205	0.205	0.205
2	0.215	0.415	0.410
3	0.188	0.603	0.602
4	0.208	0.811	0.795
5	0.198	1.009	1.000

TABLE II

	Gravimetric weight Mg	$\frac{d_2}{d_1} \cdot 2.5 \cdot 1.35 \cdot A \cdot 1.6 \cdot 10^4$
1	0.300	0.306
2	0.323	0.316
3	0.310	0.308
4	0.510	0.457
5	0.450	0.442
6	0.300	0.323
7	0.390	0.373
8	0.410	0.427
9	0.330	0.331
10	0.310	0.326

1.6 = Density of "Parlodion"

The lower range for the method as described is 0.25 μ where the d_2 value approaches 0.05 (89% T). If a 1% "Parlodion" solution is used in the preceding procedure, the lower limit is about 0.1 μ with the d_2 value remaining equal to that obtained with the 2% solution as the dye would be concentrated in one-half the volume of "Parlodion". A "Parlodion" concentration of less than 1% cannot be used as the dye will form crystals in the foils. In using this colorimetric method for determing thickness one must be aware of the relative analytical error caused by an absolute photometric error of 1%[6]. Best results in absorption measurements will be obtained in the 20–60% transmission range. Other dyes giving greater extinction could be investigated, but the method using D & C green #6 is accurate and easily adapted for thicknesses (0.3–5 μ) required in historadiography.

For mass determinations on some tissues it may be necessary to have a step-wedge constructed of thick foils. To prepare thick foils the "Parlodion" concentration is increased to 3 or 4%. Due to the increased viscosity, slides dipped into these solutions will be much thicker than those dipped into a 1% "Parlodion" solution. The concentration of dye in the "Parlodion" decreases as the "Parlodion" concentration increases if the procedure described is followed, therefore the extinction for thick foils may not become too great. For very thick foils it may be necessary to change the dye saturated

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amyl acetate: amyl acetate ratio from 7:3–6:4. By varying the "Parlodion" concentration, dye concentration, or the number of times a slide is dipped and dried, a wide range of foils may be measured under conditions giving a minimum of photometric error.

A reference system having 4 steps in the wedge was constructed and exposed at 1.5 kv, 40 ma, 55 seconds in the apparatus described by CLEMMONS AND APRISON[7]. Fig. 5 is the calibration and working curve constructed by plotting the extinction (635 mμ) of the dipped foil against $(1/D_H - 1/D_S)$. $1/D_H$ and $1/D_S$ are reciprocal densities of the steps on the wedge and the supporting foil as recorded on the historadiograph. Table III gives average values for data required to plot the lower curve (Fig. 5). An unknown structure on the historadiograph having the value D_u may be interpreted in terms of d_u and mass by taking $(1/D_u - 1/D_S)$ and using the calibration curve and equations 8 and 10.

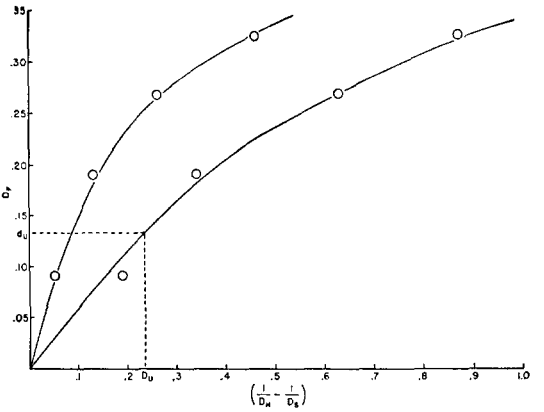

Fig. 5. Calibration Curves. Upper curve taken at 3.0 kv. Lower curve with the same foils is exposed at 1.5 kv.

$k_{H_{ref}}$ and k_{H_p} are correction factors for hydrogen in the reference system and protein. $\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}$ is the absorption coefficient for the reference system. After determining the elemental composition of the dyed "Parlodion", the absorption coefficient is calculated. As the concentration of dye in the "Parlodion" depends on the concentration of "Parlodion" in the dye-amyl acetate solution it is necessary to determine $\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}$ for the foils prepared from each stock solution that slides are dipped into.

TABLE III

Step #	D_F (655 mμ)	$1/D_H$	$(1/D_H - 1/D_S)$
1	0.08	1.70	0.19
2	0.18	1.85	0.34
3	0.27	2.14	0.63
4	0.33	2.38	0.87

$1/D_S = 1.51$

The mass of the reference system is given by the following equation:

$$m_r = \varrho\text{ref} \cdot \frac{l_1}{d_1} \cdot \frac{c_1}{c_2} \cdot d_2 \cdot K \cdot A \qquad (6)$$

A = area (1 cm²) ϱ ref = density of "Parlodion"

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Combining equation (6) with ENGSTROM's equation for the determination of mass (m_a) of the biological structure.

$$m_a = \frac{k_{H\text{ref}}}{k_{Hp}} \cdot \frac{\left(\frac{\mu}{\varrho}\right)_{\text{CNO}_{\text{ref}}}}{\left(\frac{\mu}{\varrho}\right)_{\text{CNO}_p}} \cdot \varrho \text{ ref} \cdot \frac{l_1}{d_1} \cdot \frac{c_1}{c_2} \cdot A \cdot K \cdot d_u \tag{7}$$

d_u is obtained from the calibration curve. $d_u = d_2$ as the unknown structure measured equals mass of a step in the wedge as illustrated in Fig. 5.

$$d_u = \tan\theta \left(\frac{1}{D_u} - \frac{1}{D_S}\right) \tag{8}$$

$$\tan\theta = \text{slope (Fig. 5)}$$

$1/D_S$ = reciprocal of the supporting foil radiographic density.

Equation (8) can be combined with (7).

$$m_a = \frac{k_{H\text{ref}}}{k_{Hp}} \cdot \frac{\left(\frac{\mu}{\varrho}\right)_{\text{CNO}_{\text{ref}}}}{\left(\frac{\mu}{\varrho}\right)_{\text{CNO}_p}} \cdot \varrho \text{ ref} \cdot \frac{l_1}{d_1} \cdot \frac{c_1}{c_2} \cdot A \cdot K \tan\theta \left(\frac{1}{D_u} - \frac{1}{D_S}\right) \tag{9}$$

The equation is simplified by substituting the values for the constants and is applicable only to the linear portion of the curve.

$$m_a = R \cdot K \cdot \tan\theta \left(\frac{1}{D_u} - \frac{1}{D_S}\right) \tag{10}$$

SUMMARY

A simplified method for preparing the reference system used in quantitative historadiography has been described. Glass slides are dipped into a standard dye-Parlodion-acetate solution and then dried. Squares are cut on the dried surface and the foils stripped from the slide by immersing it into water. The foils are lifted onto the preparation disc, one at a time and a wedge constructed. By measuring light absorption of the dyed wedge it is possible to calculate foil thickness. The procedure is quick, accurate, and requires no apparatus other than the microdensitometer used for density measurements on the historadiograph.

RÉSUMÉ

Les auteurs décrivent une méthode simplifiée de préparation d'un système de référence pour l'historadiographie quantitative. Des lames de verre sont plongées dans une solution d'acétate de Parlodion et d'un colorant standard, puis séchées. On découpe des carrés sur la surface sèche et l'on détache de la lame les fragments obtenus par immersion dans l'eau. Les fragments sont placés un par un sur le disque à préparation, de façon à former une pile. En mesurant l'absorption de la lumière à travers la pile colorée, on peut calculer l'épaisseur de chaque fragment. Cette méthode est rapide, précise et n'exige pas d'autre appareillage que le micro-densitomètre employé aux mesures de densité en historadiographie.

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ZUSAMMENFASSUNG

Eine vereinfachte Methode zur Herstellung des in der quantitativen Historadiographie benützten Bezugssystems wurde beschrieben. Glas-objektträger werden in eine geeichte Farbstoff-Parlodion-acetat-Lösung getaucht und dann getrocknet. Dann werden Quadrate auf der getrockneten Oberfläche geschnitten und die Folien vom Objektträger durch Eintauchen in Wasser abgezogen. Die Folien werden eine nach der anderen auf die Präparationsscheibe gebracht und ein Keil zusammengesetzt. Durch das Messen der Lichtabsorption des gefärbten Keils ist es möglich, die Dicke der Folien zu berechnen. Das Verfahren ist schnell und genau und erfordert keine weitere Apparatur als das Mikrodensitometer welches zur Dichtebestimmung auf dem Historadiograph verwendet wird.

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