PROCEDURES AND ERRORS IN QUANTITATIVE HISTORADIOGRAPHY*

by

JACKSON J. CLEMMONS

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

Historadiography is a term that was used by LAMARQUE[1,2] to describe the use of very soft X-rays (2–10 A) for the study of structures with microscopic dimensions. Animal and plant tissues a few microns thick were X-rayed at potentials of 5 kV in a special apparatus, and the historadiograph was recorded on very fine-grain Lippman film capable of magnification up to 500 times. LAMARQUE's procedures were an improvement over those of other workers[3,4] in the field and provided a new method for morphological studies and a method for determining the localization of pharmaceuticals containing heavy metals[5]. ENGSTROM AND LINDSTROM[6] greatly advanced the use of the technique in histochemistry by applying physical laws dealing with X-ray absorption. This advancement enabled quantitative *in situ* determinations of the mass of various histologic and cytologic structures. The technique is new and has had serious limitations to overcome in order to be of more than semiquantitative value. A discussion of the procedures developed in this laboratory and their limitations and errors will be presented.

Theory

X-rays are absorbed according to the Lambert-Bouguer law. I_{x_0} is the intensity of

$$-\log_e \frac{I_{x_1}}{I_{x_0}} = \left(\frac{\mu}{\varrho}\right) \cdot \varrho \cdot X \tag{1}$$

the initial X-ray beam and I_{x_1} the intensity of the beam after passing through an absorbing substance of thickness X and density ϱ. If the beam of radiation has a cross section of 1 cm^2, μ represents the fraction of energy absorbed per cubic centimeter of the absorber traversed. The mass absorption coefficient (μ/ϱ) is used as it enables an expression of the absorption per gram of the absorber.

$$\varrho_x \cdot X = m_x \tag{2}$$

m_x is the mass of the absorber per square centimeter.

$$d_x = -\log_{10} \frac{I_{x_1}}{I_{x_0}}$$

$$d_x = \left(\frac{\mu}{\varrho}\right)_x \cdot \frac{m_x}{2.3} \tag{3}$$

X-rays differ from visible light in that their absorption is an atomic process, and the mass absorption coefficient is in an additive relationship to those of the elements present.

* Aided by grants from the Atomic Energy Commission AT(11-1)-64 Project 8.

References p. 321.

If the sample is a protein with the composition carbon, hydrogen, nitrogen, oxygen, and unknown elements in the proportion C, H, N, O, and R, equation (4) represents the general case where R could represent other than the principal elements.

$$C + H + N + O + R = 1$$

$$\left(\frac{\mu}{\varrho}\right)_p = \left(\frac{\mu}{\varrho}\right)_C + \left(\frac{\mu}{\varrho}\right)_N + \left(\frac{\mu}{\varrho}\right)_H + \left(\frac{\mu}{\varrho}\right)_O + \left(\frac{\mu}{\varrho}\right)_R \tag{4}$$

In order to determine the mass of a histologic structure by X-ray absorption, a comparison is made between the absorption of the X-rays by a reference of known weight per μ^2, m_r, and that of the unknown structure, m_p. The weight per μ^2 of the unknown is converted to mass per μ^3, M_p, on dividing by the structure's thickness.

$$d_p = \left(\frac{\mu}{\varrho}\right)_p \cdot \frac{m_p}{2.3} \tag{5}$$

$$d_r = \left(\frac{\mu}{\varrho}\right)_r \cdot \frac{m_r}{2.3} \tag{6}$$

$(\mu/\varrho)_p$ and $(\mu/\varrho)_r$ are the mass absorption coefficients for the protein and the reference material.

Dividing equation (5) by (6) and solving for m_p:

$$m_p = m_r \frac{\left(\frac{\mu}{\varrho}\right)_r}{\left(\frac{\mu}{\varrho}\right)_p} \cdot \frac{d_p}{d_r} \tag{7}$$

and when the thickness of the standard is adjusted to absorb X-rays to the same extent as the unknown:

$$m_p = m_r \frac{\left(\frac{\mu}{\varrho}\right)_r}{\left(\frac{\mu}{\varrho}\right)_p} \tag{8}$$

The relationship is greatly simplified if the protein and reference have identical composition.

$$m_p = \frac{d_p}{d_r} \cdot m_r \tag{9}$$

Equation (9) is seldom the case, for the following reasons: the variation in composition of different proteins; the extreme difficulty in preparing thin protein standards which necessitated the use of a nitrocellulose for the reference system; the polychromatic X-ray beam.

When using the nitrocellulose reference system, equation (8) expresses the general case, as the mass absorption coefficient of the protein and the reference material have different values. ENGSTROM AND LINDSTROM[6] based their mass determination on a calculation of CNO mass absorption coefficients $(\mu/\varrho)_P$CNO and $(\mu/\varrho)_R$CNO. These values were calculated from the percentages of carbon, nitrogen, and oxygen obtained by a chemical analysis of the reference material and an average protein. The mass absorption coefficient for a CNO protein and reference system was obtained by substituting values for the elemental mass absorption coefficients at 8 A and the relative amounts of carbon nitrogen, and oxygen into equation (4). It was then necessary to introduce the corrections k_{Hp} and k_{Hr} for the hydrogen content of the protein and the reference material.

References p. 321.

A systematic error arising from deviations from the assumed composition of an average protein has been considered by ENGSTROM AND LINDSTROM[6]. Equation (10) was used by these investigators for the calculation of mass.

$$m_p = \frac{\left(\frac{\mu}{\varrho}\right)_R^{\text{CNO}}}{\left(\frac{\mu}{\varrho}\right)_P^{\text{CNO}}} \cdot \frac{k_{\text{H}_p}}{k_{\text{H}_r}} \cdot m_r \tag{10}$$

$$m_p = B \cdot m_r \tag{11}$$

The above discussion and calculations are based upon the absorption of monochromatic X-rays (8 A), although the X-ray beam is polychromatic, as is illustrated in Fig. 1. A method will be discussed for the determination of "mass absorption constants", Z_P and Z_R, for the protein and the reference material under the exact conditions of the historadiographic procedure. The technique and the calculations are thus simplified, and the accuracy of the method is increased.

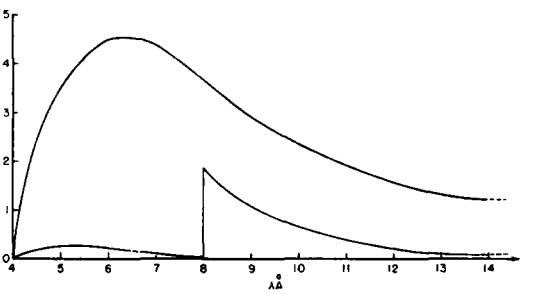

Fig. 1. Characteristic X-ray beam. Top curve indicates the variation of intensity with wavelength at 3 kV tube potential. Bottom curve indicates the effect of a 9μ aluminum filter on radiation.

The X-ray beam in the historadiographic procedure has its greatest intensity between 8–12 A. It is necessary to define a "mass absorption constant", Z, for this spectra instead of the mass absorption coefficient which is defined for monochromatic X-rays only. The mass absorption coefficient remains constant for a given wave length with changes in the mass per unit area of the absorber. The "mass absorption constant", Z, for the broad spectrum of X-rays may be relatively constant for only a limited range of mass variation. For an accurate calculation of mass it is necessary to determine experimentally the "mass absorption constant" for an average protein or other material for analysis and the reference material. When these constants have been determined, equation (10) becomes modified, and the calculation of mass is made by using equation (12).

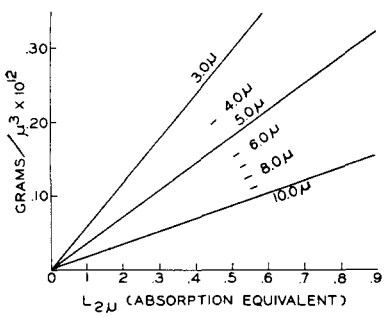

Fig. 2. Graph for converting absorption equivalent to concentration at different section thickness. (2 kV)

$$m_p = \frac{Z_R}{Z_P} \cdot m_r \tag{12}$$

The ratio of the mass absorption constants defines a constant B.

$$m_p = B \cdot m_r \tag{13}$$

B has the same significance in equations (11) and (13) but was derived by different methods.

The unknown on a historadiograph absorbs to the same extent as a reference foil of thickness l_2, the "absorption equivalent". The value l_2 is convenient to work with and will be used instead of mass per unit area in the text of this paper. The conversion from m_p to mass or grams of protein per μ^3, M_p is made with equation (14) or Fig. 2.

$$M_p = \frac{l_2 \cdot \varrho_r \cdot B \cdot 10^{-12}}{l_s} \tag{14}$$

References p. 321.

ϱ_r is the density of the nitrocellulose reference material (1.59). l_s is the thickness of the tissue section. The experimental determination of B will be discussed.

Selecting the wave length region

The magnitude of the mass absorption coefficient varies with wave lengths, generally increasing at longer wave lengths, unless an absorption discontinuity for one of the elements is encountered. It is possible to compute the mass absorption coefficient for a substance of known composition if the wave length region is known. With this value it is possible to determine the degree of transparency of the absorber to the X-rays and then vary the wave length to obtain the desired absorption for greatest accuracy in the mass determination. As mentioned previously, monochromatic X-rays are not used in historadiography but a continuous spectrum that is defined by varying the tube potential and through the use of filters. In order to estimate the transparency of the specimen, it is essential to have an understanding of intensity and wave length variation as a function of the tube potential, tube current and thickness of filters.

If the X-ray tube is operated at a given potential, the highest energy (shortest wave length) X-rays have a wave length that can be calculated using equation (15).

$$\lambda_0 = \frac{hc}{eV} \tag{15}$$

e = charge on an electron ($4.80 \cdot 10^{10}$ e.s.u.)
c = velocity of the radiation ($2.99 \cdot 10^{10}$ cm/sec)
h = Planck's constant ($6.55 \cdot 10^{-27}$ erg sec)

The above constants may be combined to give the minimum wave length, λ_0, in Angstrom units for any tube potential, V, in kilovolts.

$$\lambda_0 = \frac{12.340}{V} \tag{16}$$

Knowledge of the minimum wave length enables the Kulenkampff equation (17) to be used for calculating the shape of intensity curves (Fig. 3) for the various tube potentials.

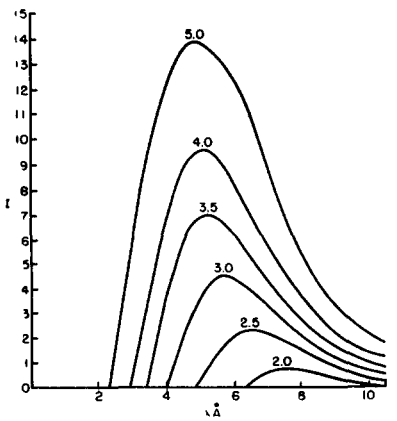

Fig. 3. The continuous X-ray spectrum for tube potentials between 2 to 5 kV.

$$I = \frac{CZ}{\lambda^2}\left(\frac{1}{\lambda_0} - \frac{1}{\lambda}\right) + bZ^2\frac{1}{\lambda^2} \tag{17}$$

b and C are constants, and Z is the atomic number of the target element. b is so small that the second term is generally neglected.

The mass absorption coefficient, at various wavelengths, for aluminum is obtained from physical tables. These constants permit calculation of the transmittance for various thicknesses of aluminum. By correlating this information with the intensity curves (Fig. 3) it is possible to indicate the effect of a 9μ aluminum foil on the continuous spectrum of X-rays produced when the tube potential is 3 kV (Fig. 1). The filter effectively reduces the intensity of the spectrum below 8 A, the point at which aluminum has an absorption discontinuity. The net result is a spectrum with maximum intensity at 8–10 A. Higher potentials are

References p. 321.

required for thin sections of bone. For thin sections of tissue, originally containing a high water content, it may be necessary to use potentials as low as 1.0–2.0 kV. When potentials as low as 1.0 kV are used, the 9μ aluminum filter has no value in defining the X-ray beam and only reduces the intensity. For very low voltages, thin Parlodion films may be shadowed with aluminum. This filter absorbs the visible cathode light, but is very transparent to X-rays.

Preparing the sample for analysis

Samples for analysis by historadiography are preferably fixed by the freeze-dry technique[7,8]. TURCHINI[9] discussed some of the advantages and disadvantages of various fixation procedures. The description appeared before the technique was introduced as a quantitative procedure, and therefore some of the methods cannot be used. Shrinkage, loss of cellular components, diffusion, and the presence of heavy metals in the fixative are to be avoided in the quantitative technique. Subsequent treatment required for other histochemical tests will also influence the choice of method in tissue fixation. Paraffin embedding is generally used, but the carbowax technique as described by RINEHART AND SULEIMAN[10] has been used when determining the lipid content of structures. The tissues are sectioned (4–10μ) and floated on water or mercury and then lifted onto the supporting foil of a disc (Fig. 4). Occasionally it is advisable to place the section directly on the disc. The samples are placed in an oven at 45° C for 20–30 minutes before removing the embedding medium.

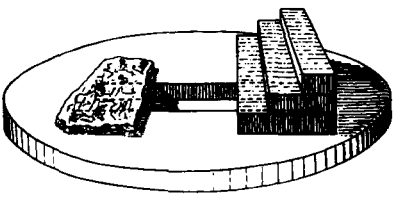

Fig. 4. Foils and specimen are mounted on a metal disc. Illustration is not in proportion, as the thickest foil is generally 0.1 to 0.5 the thickness of the tissue.

Thickness of section

The X-ray procedure does not damage the tissue, and therefore it may be stained or used for other histochemical studies. If, for example, the mass of a nucleus is determined and then the DNA content determined by other methods, the per cent DNA of the total weight of the nucleus may be expressed without knowledge of section thickness. In experiments where relative values of mass suffice, it is advisable to embed the control and experimental tissue in the same paraffin block. The simultaneous sectioning of the samples may eliminate the need for thickness determinations. However, for some problems a thickness determination is required. This has been a difficult problem in histochemical studies, and various methods have been described to overcome it. Interferometry[11], optical micrometers[12], microcators[13] are among the procedures in use and have been reviewed by LANGE AND ENGSTROM[14]. A method has been in use in this laboratory that gives an average thickness for the entire section but not a measurement of individual structures.

The procedure involves a determination of the mass of a standard protein plug that is embedded next to the tissue so that the two are sectioned simultaneously. The standard is prepared from the following solutions:

(a) 3% fibrinogen in normal saline
(b) 10% albumin in normal saline
(c) 5 mg thrombin in 10 ml saline.

References p. 321.

The solutions are centrifuged to remove any undissolved material. Five ml of the albumin solution are mixed with 4 ml of the fibrinogen solution, 1 ml of the thrombin solution is added, and the clot which forms is allowed to remain overnight at 5° C. The clot is cut into small pieces and freeze-dried, or fixed in acetone for 48 hours, and then placed in 10% formalin for further fixation and hardening. The preparation is then treated as any tissue and finally embedded in paraffin. The clot is removed after it is thoroughly impregnated with paraffin and then cut into squares so that no excess of paraffin remains on the surfaces. The volume of these pieces is determined by Archimedes' principle, and the sample is then thoroughly deparaffinized and weighed. The weight per unit volume for each preparation is calculated. This preparation serves as a standard and is embedded next to the tissue, and the two are sectioned simultaneously. A calibration curve of thickness *vs.* mass per μ^2 is constructed. This is easily done, as the density of the preparation was determined as outlined above. When a section is cut, the tissue is placed on one disc and the standard on another and historadiographs taken. The mass of the standard is determined and from this the thickness of the section.

The historadiographic apparatus

A special apparatus is required for quantitative historadiography, as the extremely soft X-ray region (8–12 A) is used. The specimen and film are placed in a vacuum chamber to avoid absorption of the soft X-rays by air. LAMARQUE[2] designed an instrument consisting of a demountable, continuously evacuated X-ray tube separated from a photographic chamber by a thin lithium window. As the photo chamber is at a very low pressure and the window thin, there was little absorption of the X-rays. The apparatus produced good radiographs when operated at 7–10 kV and 80–100 mA. Exposure times were 10–45 minutes. There is a marked increase in the accuracy of the technique if lower potentials are used in the quantitative technique. ENGSTROM[6, 15] has described two instruments, one suitable for bone or tissues impregnated with heavy metals and the other designed for soft tissues. A modification of this apparatus is presented by BRATTGARD *et al.*[16]. To facilitate work with the technique, CLEMMONS AND APRISON[17] introduced electrostatic focusing and a vacuum lock. With this apparatus 10–15 exposures may be made per hour with potentials as low as 1 kV. An accessory to this apparatus has been devised to obtain experimentally the values Z_P and Z_R, thereby eliminating the need for a tedious chemical analysis of the reference and an average protein, and the introduction of correcting factors for hydrogen (Figs. 5 and 6). The determinations are made under conditions identical with the mass measurements, and the mass absorption constants are for the polychromatic beam. The accessory apparatus and its application will be discussed under photometric error.

The above instruments have no primary magnification and are limited by the resolving power of the Lippman film. Although not developed, as yet, for quantitative historadiography, two instruments of considerable interest have been described. COSSLETT AND NIXON[18] have developed an X-ray shadow microscope in which a micro-focus X-ray tube gives a point source of X-rays. By placing a specimen at a given distance from the source, a primary enlargement of up to 300 × with resolution of 1μ is obtained. The instrument has only been used with dense structures or material impregnated with heavy metals. Voltages up to 15 kV are used, and the film and sample are not placed in a vacuum chamber. This technique must be modified to meet the requirements for quantitative mass determinations of structures in thin tissue sections; however, it may offer

References p. 321.

possibilities in overcoming the limitation in resolution when working with the sample in direct contact with Lippman film.

KIRKPATRICK AND BAEZ[19] have developed an X-ray microscope capable of high resolution, but this instrument has not been applied to thin tissue sections for histological investigations.

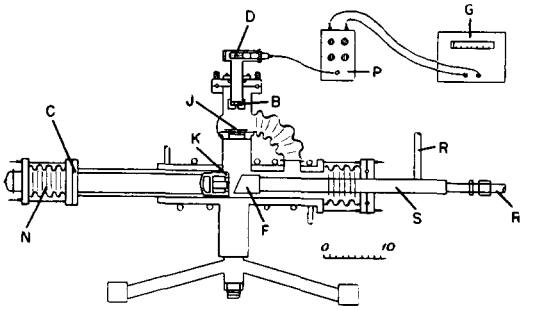

Fig. 5. Frontal section through historadiographic apparatus equipped with a crystal detector. B – Cesium bromide crystal; C – Cathode; N – Metal bellows; D – Photomultiplier tube; F – Anode; R – Inlet and outlet for water cooling; G – Galvanometer; J – Aluminum filter (9μ); K – Focusing cup; P – Photo-tube power supply.

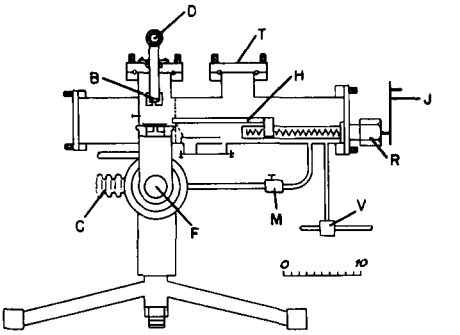

Fig. 6. Section through photographic chamber of historadiographic apparatus equipped with crystal detector. B – Cesium bromide crystal; C – Outlet to vacuum system; D – Photomultiplier tube; T – Port for inserting camera; H – Slide for carrying camera through vacuum lock; F – Anode; V – 3-way valve; R – Rotary vacuum seal; J – Crank for moving sample.

The supporting foil

The tissue and reference system are mounted over a slit in a metal disc made from brass or stainless steel. It is necessary to cover the slit with a very thin supporting foil. The discs are coated with a 1:1 albumin-glycerine solution, allowed to dry, and are then placed on a wire rack in a funnel-like container (Fig. 7). Water is added to a level above the rack and all particles removed from the surface. A few drops of a standard 1% Parlodion-dye-acetate solution, as was used in preparing a reference wedge, are added to the surface and allowed to spread evenly. As the Parlodion film begins to shrink, the water level is lowered until the film is in contact with the metal discs. The rack and discs are dried in an oven at 45° C. The discs are separated and examined after they have dried. Discs possessing multicolored interference patterns or artifacts are discarded.

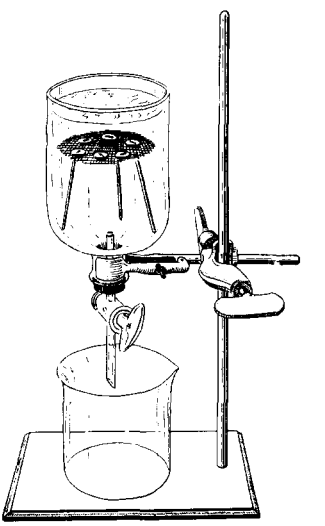

Fig. 7. Apparatus for preparing supporting foils.

A source of error frequently overlooked in this technique is the non-uniformity of supporting foils. If this membrane is prepared as previously described with the use of a standard 1% Parlodion-dye-acetate solution containing D and C green #6, as used in the reference system, it is possible to determine the thickness of the membrane by using equation (22). The value ΔT_F is obtained, and from equation (24) the per cent variation in thickness, $\Delta l_r/l_r$ may be calculated. If this value is too great, the supporting foil should be discarded, as the unevenness will produce a large error in

References p. 321.

the mass determination. Omission of this step may lead to errors of 25–50% when the tissues analyzed have small mass and require reference foils 0.3–0.5 μ thick.

The reference system

The purpose of the reference system has already been discussed. Several methods for preparing and measuring the thickness of thin nitrocellulose foils required for this system have been described[20, 21, 22]. In this laboratory, the method described by CLEMMONS AND WEBSTER[23] is in use. Thin, uniform foils are prepared by immersing glass slides into a standard solution of Parlodion and D and C green # 6* in amyl acetate. After the slides have dried, cut foils are stripped from them by immersion into water (Fig. 8). The thickness of each step forming the reference wedge is calculated by measuring its extinction at 635 mμ. The procedure requires a comparison, at this wavelength, between a standard Parlodion-dye-acetate preparation in a 1 cm cuvette measured with a Beckman spectrophotometer and the mounted reference foil, whose extinction is measured with a microcolorimeter. Equation (18) was derived for calculating thickness of the foils by this method.

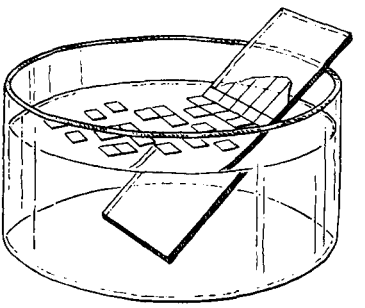

Fig. 8. The cut foils are stripped from the glass slide by immersing it into water.

$$l_2 = \frac{d_2}{d_1} \cdot \frac{C_1}{C_2} \cdot K \tag{18}$$

C_1 and C_2 represent the concentrations of Parlodion in the standard solution and in the reference foil. d_1 is the extinction for the standard solution, and d_2 for the foil. K is a constant correcting for the weight of the dye that was added to the Parlodion. K also corrected for differences in the half band-width characteristics of the two instruments. The procedure can be further simplified from the following relationship:

$$\frac{d_s}{d_2} = \frac{C_s l_s}{C_2 l_2} \tag{19}$$

d_s is the extinction for a standard foil of thickness, l_s, having concentration C_s; l_2, C_2 and d_2 are values for the unknown foil as defined above. C_s and C_2 are equal, as the weight per unit volume of the foils is independent of foil thickness.

$$l_2 = \frac{l_s}{d_s} \cdot d_2 \tag{20}$$

As the Lambert-Beer law can be applied to the system, a new constant, K_N, can be defined, and equation (20) reduces to (22).

$$K_N = \frac{l_s}{d_s} \tag{21}$$

$$l_2 = K_N \cdot d_2 \tag{22}$$

$$l_s = \frac{w}{\varrho_r \cdot A} \tag{23}$$

w = weight of the standard foil
ϱ_r = density of the foil material
A = area of the standard foil.

* National Aniline Division Allied Chemical and Dye Corp., New York 6, N.Y.

References p. 321.

The concentration of dye in the nitrocellulose foils can be varied by altering the amount of Parlodion* used in preparing the standard Parlodion-dye-acetate solution. The concentration of dye in the acetate solution is constant. When a small amount of Parlodion is added to the dye-acetate, evaporation of the acetate leaves a high concentration of dye in the Parlodion; the opposite occurs when a large amount of Parlodion is used. The Parlodion alters the viscosity of the dyed solution, and the viscosity determines to an extent the thickness of the Parlodion foils formed by immersing slides into the Parlodion-dye-acetate solution. Fig. 9 gives the extinctions obtained for foils of varying thickness prepared by using 1–4% concentrations of Parlodion in acetate.

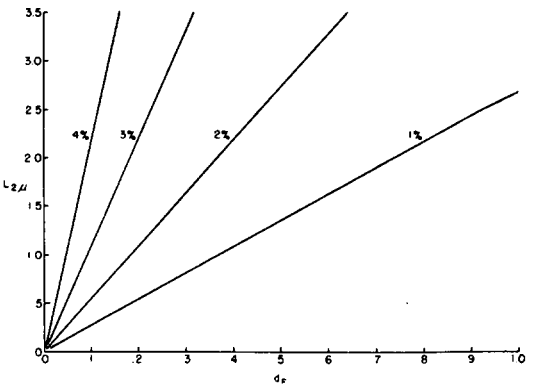

Fig. 9. Graph relating thickness and extinction of dyed foils prepared from 1–4% Parlodion.

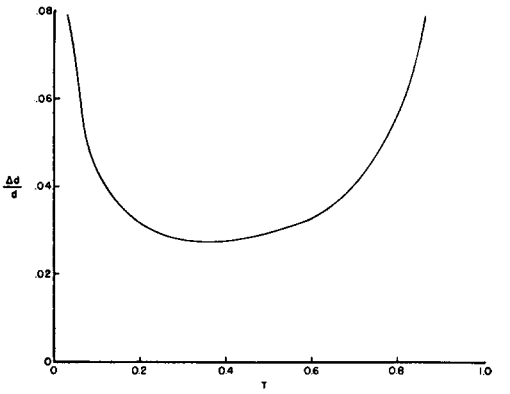

Fig. 10. Error in extinction for values of transmittance assuming the uncertainty in T is 0.01.

The accuracy of the method is primarily dependent upon the reliability of the photometry in determining d_2 with the microcolorimeter. In arriving at this photometric error, the relationship between transmittance, T, and extinction, d, is considered [24,25].

$$\frac{I}{I_0} = T \qquad d = -\log_{10} T$$

$$\frac{\Delta d}{d} = \frac{\Delta T}{Td} \cdot 0.434 \tag{24}$$

The fractional error in d for any uncertainty ΔT in determining T is given by equation (24). ΔT is obtained by taking several readings of T for the same foil, and may be taken as the standard deviation of the measurement. In most cases, ΔT may be the inaccuracy in reading the transmittance scale (0.01–0.005). Figs. 9 and 10 may be used together to determine the uncertainty of the d value for foils of varying thickness.

The probable error in the thickness of a foil as calculated in equation (22) is given in equation (25).

$$\Delta l_2 = [(d_2 \Delta K_N)^2 + (K_N \Delta d_2)^2]^{1/2} \tag{25}$$

Δd_2 is calculated from equation (24).

ΔK_N may be determined from the experimental determination of K_N. Conditions may be selected so that the error in K_N is minimized (1–2%). This is done by having a standard foil thick enough to give an accurate determination of d_s and one that can be easily weighed with an accurate microbalance. The only significant error results in determining the thickness of very thin foils ($< 0.5\ \mu$) when the transmittance of the foils is high. It is seldom that reference foils of less than $0.3\ \mu$ are required. The error for this

* Mallinckrodt Chemical Works, New York, N.Y.

References p. 321.

thickness, using a 1% standard dye-Parlodion-acetate solution, is only 3–5%. The colorimetric method for thickness measurements can be used for foils as thin as 0.15 μ. There are interferometric methods that can be used to measure the thickness of extremely thin foils, but great accuracy in measuring foil thickness does not increase the accuracy of the mass determination. If the mass absorption coefficient for the reference material is 1090 at 8 A, a foil 0.15 μ has a transmittance to the X-rays of 0.97, and on increasing the thickness 100%, the transmittance is only reduced to 0.95. A high degree of accuracy in measuring the thickness of the extremely thin foils will not overcome the photometric error in determining the mass of a structure with very high transmittance to X-rays.

DJURLE AND HALLEN[26] have described an interferometric method for determining the thickness of nitrocellulose foils. The uncertainty of the method was stated to be 0.028 μ ($\Delta l_2 = 0.028$) and the authors give this value as 1% of thinnest foils that they used. This would indicate that the thinnest foils used by these workers were 2.8 μ. The water content of embedded tissues has been replaced by paraffin, which is removed prior to taking a historadiograph. A 5 μ section of tissue originally containing 50% protein and 50% water would require a reference foil approximately 1.3 μ thick. This would indicate that DJURLE AND HALLEN have represented the error in measuring foils used for tissues possessing a very high protein content, or for tissue sections greater than 10 μ thick. The error of the colorimetric method is approximately 5% for foils 0.1 — 0.2 μ thick, compared with an error of 15–25% for DJURLE AND HALLEN's method when used in the same range. Interferometric methods of greater accuracy are available, but, as discussed previously, these methods will not increase the accuracy of the historadiographic procedure, as the uncertainty in determining the X-ray absorption for very thin foils becomes the limiting factor.

The colorimetric method is accurate and provides a quick means for scanning the supporting foils and each step of a reference wedge in order to detect any non-uniformity in thickness. The measurements of d_2 are made after the foils are mounted, as illustrated in Fig. 4. It is possible to have direct recordings of readings across the wedge when the microcolorimeter is used with a motor driven stage and a recording galvanometer. Another advantage of the colorimetric procedure for thickness measurements is that the microdensitometer used for reading the historadiographs is easily converted to a microcolorimeter and this eliminates the need for additional expensive apparatus.

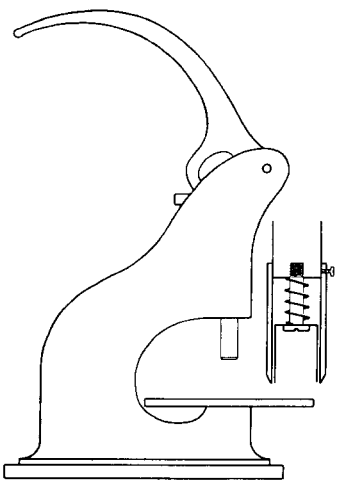

Fig. 11. Punch for cutting small discs of film.

Developing the historadiograph

Discs of film (15 mm in diameter) are punched from 6 × 9 cm sheets of film with an altered riveting machine (Fig. 11). Extreme care in handling the film is necessary to avoid dust particles, scratches, and finger prints. The metal disc, with tissue and reference wedge, is placed in direct contact with the emulsion side of the fine-grain film and then exposed to X-rays. The historadiographs are developed in filtered solutions and then dried under dust-free conditions. The films can be held in a rack as illustrated in

References p. 321.

Fig. 12. A rack of this type is convenient, as it may be used to hold the preparation for staining procedures after the historadiographs have been taken.

Two types of film have been used for the historadiographic procedure. Gevaert-Lippman film and Eastman 649 film have been found satisfactory. ENGSTROM AND LINDSTROM[27] and BELLMAN[28] have discussed the properties of some microradiographic films. The developer recommended for the Lippman film is the following:

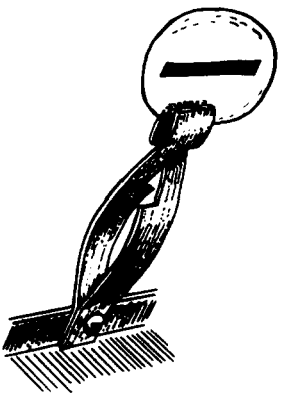

Fig. 12. Holder used in developing and drying the historadiograph.

G-209 A Pictol-Hydroquinone Developer

Pictol	4 g
Sodium sulfite (cryst.)	130 g
or anhydrous	65 g
Hydroquinone	10 g
Sodium carbonate (cryst.)	110 g
or anhydrous	45 g
Potassium bromide	5 g
Water	1000 ml

The following data apply to Lippman film:

Speed: A very slow emulsion at 5 kV; it is 300 × less sensitive than standard X-ray film.
Contrast: A gamma of 3.5 when developed for 6 minutes.
Grain: 500 linear enlargements without grain.
Developer: G-209A, develop at 18° C for 6 minutes.
Darkroom Light: Lippman film can be treated under a bright red or yellow light.

Microdensitometry

The historadiograph is placed between a glass slide and a cover glass which is then taped to the slide. The mounted film should be inspected for "Newton's rings". These rings may appear frequently and are formed by interference in the air film trapped between the historadiograph and the cover slip or slide. The colored patterns act as miniature filters, absorbing a portion of the light path through the densitometer, and therefore introduce large errors.

Direct and indirect procedures have been used for the microdensitometry of the historadiograph. The indirect method involves making a photomicrograph of the historadiograph in such a manner that the structure and the reference appear on the same photographic plate. This procedure has all of the problems encountered in photomicroscopy, although it does provide a permanent enlarged record of the structures measured. BRATTGARD *et al.*[12] state that this method has a very large error. In the direct method, a microdensitometer is used to take readings on the original historadiograph. The instrument used is similar to the microcolorimeter described by POLLISTER[29]. An infra-red absorbing filter is used to avoid melting the photographic emulsion. The details of the apparatus are illustrated in Fig. 13. When a 635 mμ filter is used, the same instrument is used to obtain extinctions of the dyed reference foils. These values are used, as explained in the discussion of the reference system, to calculate the thickness of steps in a reference wedge. The apparatus may be used as a microcolorimeter to obtain other histochemical data to be correlated with historadiographic observations.

Many readings are required of the dyed reference foils and of their image on the

References p. 321.

historadiograph in order to construct a calibration curve. To facilitate reading the wedges a motor driven stage was constructed. This device enables numerous readings to be taken quickly and is indispensable when a recording galvanometer is used. The stage is driven by a 2 RPM motor and moves from 0.5–8.0 microns per second. Frequently, small inhomogenieties exist in the emulsion and do not become apparent if only a few readings are made on a wedge. With the motor driven stage, Fig. 14, readings are taken down one portion of the wedge and up another. Manual movement of the stage is used when specific structures are to be measured. Extreme care should be taken at this point, and it is the most time-consuming operation. Centering the structure on the cross-hair of the densitometer is a tedious manipulation, as a fractional movement of the stage will move the object several millimeters in the image plane. A gear arrangement similar to that used in some electron microscopes is used for centering the object to be measured.

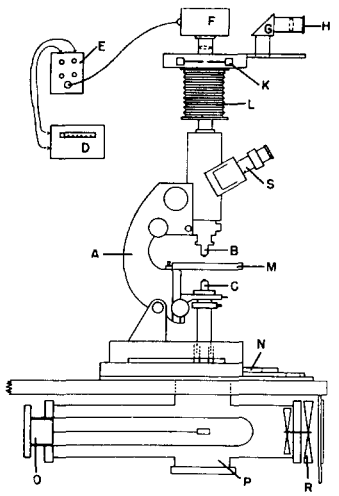

Fig. 13. Microdensitometer and microcolorimeter.
A – Microscope stand; B – Objectives on clutchmount; C – Objective for condenser; D – Galvanometer; E – Phototube power supply; F – Phototube housing; G – Prism; H – Telescope; K – Iris diaphragm; L – Bellows; M – Motor-driven stage; N – Filter holder; O – Light source (AH_4 or Ribbon filament); P – Light source housing; R – Cooling fan.

The reading of the historadiograph is done by the relative-transmittance technique. In this technique the microdensitometer is focused on the historadiographic image of the thickest step of the reference wedge (I_3 in Fig. 15). The sensitivity of the instrument is adjusted so that transmittance, T_{H3}, for this step is 1.0. The motor-driven stage is started, and the T_H readings across I_3, I_2, I_1 and I_0 are recorded automatically. Manual manipulation is then used to read the transmittance of I_s, which represents the image of the tissue specimen. A calibration curve consisting of the relative-transmittance values (T_{H_0}–T_{H_3}) plotted against the corresponding thickness of each step in the reference wedge is constructed. This plot establishes a calibration curve for determining the "absorption equivalent," l_2, of structures giving the image I_s. The "absorption equivalent" is used in equation (14) to calculate mass per μ^3. In the case cited, I_3 was set for maximum transmittance; however, I_2, I_1, or whatever step nearest the transmittance of the structure measured in I_s, is selected for maximum transmittance and the calibration curve constructed accordingly. It should be noted (Fig. 15) that the lower the mass of the object, the darker it will appear and approach I_0 on the historadiograph. Therefore in setting the transmittance of the corresponding foil image at a maximum, there will

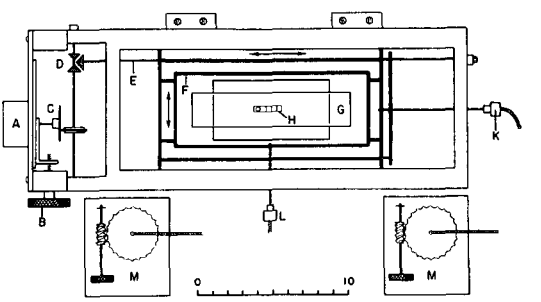

Fig. 14. Motor driven stage.
A – 2 RPM synchronous motor; B – Knob for varying speed; C – Clutch for speed control; D – Gears for changing direction; E – Threaded shaft; F – Slide carrier; G – Glass slide; H – Historadiograph; K – Shaft for horizontal movement; L – Shaft for vertical movement; M – Reduction gear.

References p. 321.

be a greater amplification factor than if the foil had been very thick. The importance of this amplification factor will be discussed with photometric errors. The relative-transmittance method is more accurate than ordinary procedures where the clear or unexposed portion of the film is set at maximum transmittance. The errors and theoretical advantages of this method will be discussed.

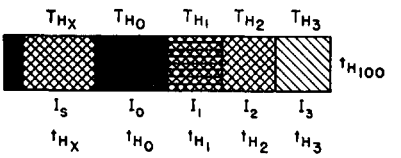

Fig. 15. The historadiographic image.

In setting I_3 at $T = 1.0$, there arises the problem of a reference point to check the densitometer for drift. Inhomogeneities exist in the film, and therefore I_3 cannot be used as a reference point. After I_3 is read, it is removed from the optical path and a neutral density filter inserted and the transmittance recorded. The filter is then removed and the readings on the historadiograph continued. Drift of the reference point is checked by reinserting the neutral density filter and checking the reading.

Determining the absorption constant, Z_R, of the reference system.

If direct measurements of I_{x_1} and I_{x_0} are made of X-rays through foils of known thickness, equation (26) may be used to arrive at the "mass absorption constant", Z_R,

$$-\log_e \frac{I_{x_1}}{I_{x_0}} = Z \cdot m \tag{26}$$

of the reference system. Technical problems have made the measurement of soft X-rays difficult, and therefore ENGSTROM AND LINDSTROM[6] obtained values for their constant from a chemical analysis of the reference material and the use of equation (4). The mass absorption coefficients for the elements were obtained from physical tables. A correction factor was introduced to correct for hydrogen. In this procedure the X-ray source was treated as if it were monochromatic, although the characteristics of the X-ray beam are as illustrated in Fig. 1. The mass absorption coefficient was expressed as $\left(\frac{\mu}{\varrho}\right)_R^{\mathrm{CNO}} \cdot k_{\mathrm{H}_R}$, where $\left(\frac{\mu}{\varrho}\right)_R^{\mathrm{CNO}}$ is the absorption coefficient for a CNO reference and k_{H_R} is the correction for the hydrogen content of the reference material.

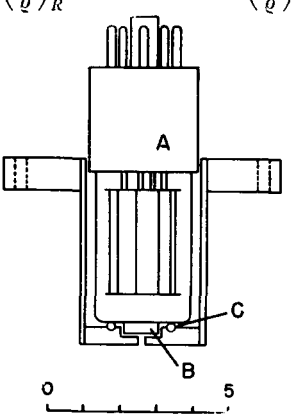

Fig. 16. Crystal X-ray detector.
A – End-window photomultiplier; B – Cesium bromide crystal; C – "O" ring seal.

In this laboratory a modified scintillation counter has been introduced into the historadiographic apparatus described by CLEMMONS AND APRISON[17]. This accessory permits an accurate measurement of X-ray intensities, I_{x_1} and I_{x_0}, through foils of known thickness while under conditions identical to those present when recording a historadiograph. The detector consists of a cesium bromide crystal mounted at the end of a lucite rod which projects into the photographic chamber of the X-ray apparatus (Fig. 5 and 6). At the opposite end of the rod is attached an RCA-IP21 photomultiplier tube connected to a Farrand power supply and a sensitive galvanometer. Another type of detector is described in Fig. 16. In this detector an end-window photomultiplier tube is introduced into the photochamber and sealed by an "O" ring. The cesium bromide crystal is placed in direct contact with the end window. This detector is more convenient to use and has greater sensitivity.

References p. 321.

To determine the "mass absorption constant", Z_R, the X-ray beam is stabilized and the I_{x_0} reading taken; then a foil of known thickness, mounted on a special disc and camera (Fig. 17), is introduced and the I_{x_0} reading recorded. The camera is easily displaced by turning the crank "J", as illustrated in Fig. 6. The results of this procedure with varying thickness and voltage are given in Fig. 18. It is possible to calculate the "mass absorption constant," Z_R, at any potential for the reference material from these curves. In this determination there is no need to correct for hydrogen. The "mass absorption constant" arrived at by this procedure is more correct than are values obtained by chemical analysis of the reference material and the use of physical constants at a single wavelength.

A 0.73 μ reference foil has an extinction of 0.081 in the X-ray beam (2 kV 9μ Al filter). Parlodion has a density of 1.59.

$$Z_R = \frac{2.3 \times 0.081}{1.59 \times 0.73 \times 10^{-4}} = 1600$$

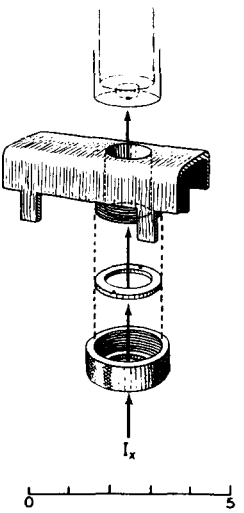

Fig. 17. Special camera and disc for use with crystal detector in the determination of Z_P and Z_R.

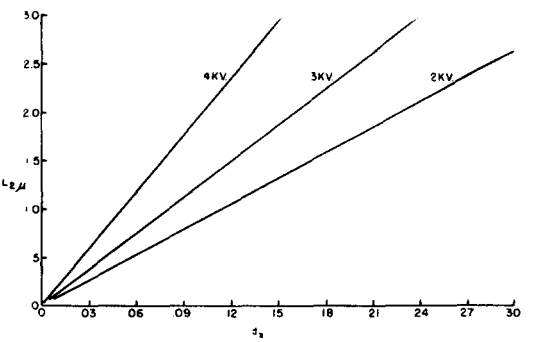

Fig. 18. Graph relating extinction and thickness for X-ray tube potentials from 2–4 kV.

Determining the mass absorption constant, Z_P, of protein

In some instances it is possible to determine the "mass absorption constant", Z_P of various proteins and other tissue components to increase the accuracy of the mass determination. An accurate estimate of these values extends the usefulness of the procedure. Albumin-fibrin clots are formed as previously described. A sample of the fixed material is analyzed for its nitrogen content to obtain a constant for converting nitrogen content to grams of protein. Sections (5–15 μ) are cut from the paraffin embedded material and mounted on 1 × 3 glass slides that have been dipped into a standard dye-Parlodion-acetate solution. One of the slides prepared for the reference system may be used. One side of the slide is cleaned with ethyl acetate so that a thin dyed nitrocellulose foil remains on one side only. The "d_2" value is obtained over a uniform area of the slide and the thickness of the foil calculated. The paraffin section of the standard is placed on this area. A 1 cm^2 area is cut in the section, pressing hard enough with the knife to cut the underlying foil. The preparation is then stripped from the glass slide by immersing it in water, as illustrated in Fig. 8. The sample is then mounted on a special disc (Fig. 17), that has been covered with a thin supporting foil of known thickness. The mounting is done on water as in the preparation of the reference system. When the sample is dry

References p. 321.

the disc is placed in the special camera (Fig. 17) and introduced into the historadiographic apparatus where I_{x_1} and I_{x_0} readings are taken with the crystal X-ray detector. Readings are taken at different X-ray tube potentials so that the "mass absorption constant" is known for different wavelength regions. The sample is then carefully removed from the disc by immersing it in ethyl acetate. The sample is washed by adding solvent, centrifuging and then decanting the solvent. This procedure is followed by a Kjeldahl digestion and nitrogen determination. The weight of the sample is calculated by using the factor determined above. The area of the sample was measured, and from the nitrogen constant, it is possible to calculate the weight, m_p, of the protein per cm^2. The I_{x_1} and I_{x_0} values are used to calculate the extinction, d_T. This value is the extinction for both the protein and the underlying nitrocellulose foils. The thickness of the underlying foil is calculated, and from Fig. 9 the extinction, d_x, of this foil is determined. The calculation of the "mass absorption constant" is made by substitution into equation (27).

$$d_T - d_x = Z_p \cdot m_p \tag{27}$$

The calculation is made for varying mass of protein. As polychromatic X-rays are being used, the value is not constant for all values of mass, although it is generally constant for the small range encountered in historadiography.

The "mass absorption constants" for other proteins or non-proteins may be determined in a similar manner. The standard is prepared as above, but a known amount of substance, X, is added to the albumin-fibrinogen solution. A nitrogen determination is made and the procedure outlined above is followed. The "mass absorption constant", Z_x, of the added substance is determined by substitution into equation (28).

$$d_T - d_x = Z_p \cdot m_T \cdot \%P + Z_x \cdot m_T \cdot \%X \tag{28}$$

In the above equation, m_T is the total weight per cm^2 of the preparation. $\%\ P$ and $\%\ X$ are known from the amounts of each used in preparing the standard.

In equation (28), X could represent bone salts, glycogen, *etc.* The determination of the constants for bone salts would be of value in studies of early ossification mechanisms. In this experiment the bone salts of one limb of an animal are extracted, precipitated and then redissolved in a known amount of the albumin-fibrinogen solution. The weight per cm^2 of the sectioned standard is determined from the nitrogen constant of the standard clot containing the added component. The d_T and d_x values are determined. In this procedure equation (28) is used, and the "mass absorption constant" is for "bone-salts" and not for any individual element or salt. The procedure is useful in determining the ratio of bone salts to protein matrix in the early stage of bone formation. During this stage of ossification it is possible to obtain thin tissue sections without decalcifying. Historadiographs may be taken of the tissue sections and the absorption of X-ray related as in equation (29).

$$d_B = Z_P \cdot m_p + Z_s \cdot m_s \tag{29}$$

Z_s is the "mass absorption constant" of the bone salts as determined with equation (28). Equation (29) represents the case before decalcification and d_B, the extinction value. Z_P is the mass absorption constant of the protein matrix and m_p and m_s the mass per cm^2 of the protein matrix and the bone salts respectively. When decalcified, the absorption of the X-rays may be expressed as follows:

$$d_A = Z_P \cdot m_p \tag{30}$$

where d_A represents extinction value following decalcification.

References p. 321.

Substracting (30) from (29):

$$d_B - d_A = Z_s \cdot m_s \tag{31}$$

It is possible to determine the ratio of bone salts to protein by the calculation of m_p and m_s, using equations (30) and (31).

The discussion demonstrates how the "mass absorption constants" may be determined and then used to gather more definite information about tissue components. In some cases the experimentally determined "mass absorption constants" for various substances will be very different. Knowledge of this is of value in ascertaining the influence a high concentration of this material will have on the total mass determination when basing calculations on the "mass absorption constant" of an average protein.

A 6.3 μ section of 20.6% protein standard absorbed X-rays (2 kV) to the same extent as a 0.73 μ reference foil.

$$m_p = \frac{0.206 \times 10^{-12} \times 6.3}{10^{-8}} = 1.3 \times 10^{-4}\ \text{g/cm}^2$$

From Fig. 18

$$\frac{l_2}{d_x} = 9 \qquad \therefore \frac{0.73}{9} = 0.081$$

$$-\log_e \frac{I_{x_1}}{I_{x_0}} = Z_p \cdot m_p$$

$$Z_p = \frac{2.3 \times 0.081}{1.3 \times 10^{-4}} = 1415$$

$$B = \frac{Z_R}{Z_p} = \frac{1600}{1415} = 1.13$$

Expression of photometric error as influenced by hardness of X-rays, mass of the absorbent, and uncertainty in photometry of the historadiograph

Photometric error may be defined as the error in mass resulting from an uncertainty in measuring the intensities of the X-ray beams (I_{x_1} and I_{x_0}). In a direct method it is the error in the direct measurement of I_{x_1} and I_{x_0} with a geiger counter or the crystal detector previously described. In the indirect or photographic recording of I_{x_1} and I_{x_0}, there is a photometric uncertainty in measuring intensities (I_{H_1} and I_{H_0}) of the visible light through the historadiograph which produces an uncertainty in measuring the X-ray intensity. In order to arrive at the photometric error in the indirect procedures one must establish the relationship between photographic blackening and X-ray intensity.

Direct measurements: In arriving at an expression of photometric error in quantitative historadiography the Lambert-Bouguer law[1] and its application to the absorption of X-rays by the nitrocellulose reference system and tissues under analysis is considered. The relationship between transmittance, T, and extinction, d, is as follows:

$$d_\lambda = -\log_{10} T_x \qquad T_x = \frac{I_{x_1}}{I_{x_0}}$$

$$\frac{\Delta d_x}{d_x} = \frac{\Delta T_x}{T_x \log_{10} T_x} \cdot 0.434 \tag{32}$$

In equation (32) $\Delta d_x/d_x$ is the error in extinction for any uncertainty, ΔT_x, in measuring the X-ray transmittance through the absorbent and may be expressed for any value of

References p. 321.

transmittance from 0–1.0. A plot of $\Delta d/d$ for values of T is given in Fig. 10. This curve has been referred to as the Twyman-Lothian curve. Since, in the direct procedure, d_x is in direct proportion to the mass of the reference system or the unknown protein mass, $\Delta d_x/d_x$ is a direct expression of the error in mass resulting from any uncertainty, ΔT_x, in measuring T_x.

$$\frac{\Delta d_x}{d_x} = \frac{\Delta m_p}{m_p} \tag{33}$$

Fig. 10 was constructed assuming ΔT equal to 0.01. It is evident that at very high or very low transmittance values the error becomes large. Low values of T_x are seldom encountered in the direct historadiographic procedure, as an increase in tube potential will increase the transmittance of the absorbant. In indirect methods, however, low transmittance values are encountered in the densitometry of the historadiograph. The transmittance for varying thicknesses of reference foils is given in Fig. 18. Fig. 19 combines data from Fig. 10 and Fig. 18 and is an expression of the error in the mass determination of an unknown structure absorbing to the same extent as foils of thickness l_2. This is the expected error in mass when there is an uncertainty of 0.01 in measuring I_{x_1}/I_{x_0} directly with a counter or similar device.

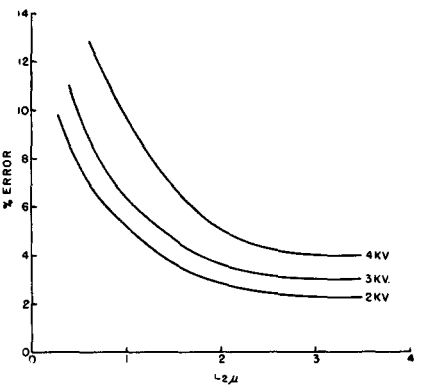

Fig. 19. Representation of the error in measuring the mass of structures having different absorption equivalents, for the 2–4 kV range.

The thickness of the reference wedge required for the analysis of a tissue may be estimated from equation (34).

$$l_2 = \frac{M_p \cdot \frac{\varrho_p}{\varrho_r} \cdot l_s}{1.59} \tag{34}$$

l_2 is the required thickness of the reference wedge, or the "absorption equivalent"; l_s is the section thickness; M_p is the protein concentration in the tissue; ϱ_r is the density of the nitrocellulose reference, and ϱ_p is the density of the average protein (1.30). A 5 μ section of tissue with protein content of 20% requires an absorption equivalent, l_2, of 0.513 μ. The protein concentration is arrived at from the wet and dry weights of fresh tissue slices. This value is only a crude approximation because of the heterogeneous distribution of water and the variability of the non-protein constituents in tissues; however, it permits an estimation of the foil thickness to be used as a reference wedge.

In Fig. 19, it can be seen that at 2 kV an uncertainty of 0.01 in measuring I_{x_1}/I_{x_0} produces a 7% error in the mass determination of a structure absorbing X-rays to the same extent as the 0.513 μ foil. The error in mass for this foil is greater than 15% when the X-ray tube potential is 4 kV. It becomes evident that there is a great advantage in using low tube potentials and even then the error for a 0.513 μ foil is above 5%. Equation (14) is used to convert the "absorption equivalent" to mass per μ^3 for any tissue section thickness. High X-ray tube potentials are used only when very thick reference foils would be required to match the absorbance of the sample.

The above discussion expresses the error for an ideal system where X-ray absorption is measured directly. A detector of the type described previously cannot be used for X-ray

References p. 321.

absorption measurements in historadiography due to the demands, as far as resolution, in measuring cellular structures. It is necessary to rely on indirect methods as recording the image on fine-grain film and subsequent microdensitometry to arrive at the values of I_{x_1} and I_{x_0}.

Indirect measurements: In the indirect method there is again interest in the density, intensity, and transmittance relationship. X-rays produce a certain opacity in the photographic film after being partially absorbed by the tissue. This opacity, O_H, is related to the transmittance of the light required to measure it in accord with equation (35).

$$\frac{I_{H_x}}{I_{H_0}} = t_{H_x} \qquad d_{H_x} = -\log_{10}\frac{I_{H_x}}{I_{H_0}} = \log_{10} O_{H_x} \tag{35}$$

The amount of metallic silver in a unit area of the image on the historadiograph is closely proportional to the photographic density, d_H. Photographic density is measured by sending a beam of light, I_{H_0}, through the area and measuring the fraction of light I_{H_x}, that emerges. The ratio I_{H_x}/I_{H_0} is called the transmittance, t_{H_x}, of the image, and the reciprocal of this is the opacity, O_{H_x}. When working with X-rays the extinction, d_H, is found to be proportional to the exposure of X-rays producing the silver deposit. Fig. 20 indicates that there is a linear relationship between the X-ray exposure and the photographic density, d_{H_x}. The shape of this curve should be determined for each pack of film. Assuming the reciprocity law for the and intensity, the following relationship is established:

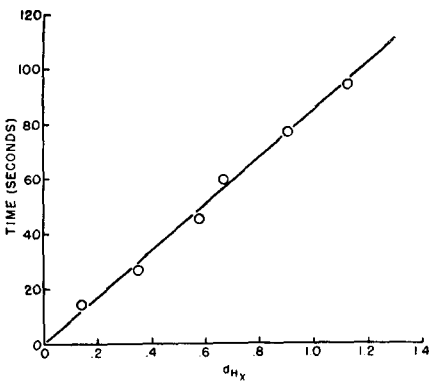

Fig. 20. Exposure and historadiographic extinctions are in linear relationship with Lippman film over the extinction range used in historadiography.

$$T_x = \frac{I_{x_1}}{I_{x_0}} = \frac{d_{H_x}}{d_{H_0}} = \frac{-\log_{10} t_{H_x}}{-\log_{10} t_{H_0}} \tag{36}$$

For direct measurements with a counter, the error in d_x for any value of T_x, and uncertainty, ΔT_x, is given by equation (32), and for indirect photographic measurements, the error in d_x is given by equation (37)

$$\frac{\Delta d_x}{d_x} = \frac{\Delta\left(\frac{d_{H_x}}{d_{H_0}}\right)\cdot 0.434}{\frac{d_{H_x}}{d_{H_0}}\log_{10}\frac{d_{H_x}}{d_{H_0}}} \tag{37}$$

$$\Delta\left(\frac{d_{H_x}}{d_{H_0}}\right) = \left[\frac{\left(d_{H_x}\frac{\Delta d_{H_0}}{d_{H_0}}\right)^2 + \left(\Delta d_{H_x}\right)^2}{{d_{H_0}}^2}\right]^{1/2} \tag{38}$$

$\Delta d_{H_x}/d_{H_x}$ and $\Delta d_{H_0}/d_{H_0}$ are errors that arise from two independent density measurements on the historadiograph and are calculated from equations (39) and (40).

$$\frac{\Delta d_{H_0}}{d_{H_0}} = \frac{\Delta t_{H_0}\cdot 0.434}{t_{H_0}\cdot -\log_{10} t_{H_0}} \tag{39}$$

$$\frac{\Delta d_{H_x}}{d_{H_x}} = \frac{\Delta t_{H_x}\cdot 0.434}{t_{H_x}\cdot -\log_{10} t_{H_x}} \tag{40}$$

References p. 321.

The value $\Delta(d_{H_x}/d_{H_0})$ may be used in equation (37) or substituted for ΔT_x in equation (32). When the latter is used, an approximation of the accuracy can be obtained by using the data in Fig. 18. Thus by knowing the approximate water content of the fresh tissue and the section thickness to be used, one may determine the region on the Twyman-Lothian curve that measurements are being made and thereby estimate the error. When an estimate of error has been made, it is possible to ascertain the gain in accuracy obtained by increasing or decreasing tube potential, or by varying the thickness of the sample.

In equation (37), $\Delta(d_{H_x}/d_{H_0})$ is the uncertainty that exists in the photographic measurement of X-ray transmittance. Δt_H in equations (39) and (40) is the uncertainty in measuring the transmittance of visible light through the historadiograph. The value for t_H may range from 0.05 to 0.80 depending upon the time of exposure to X-rays. An uncertainty, Δt_H, for values of t_H will give rise to errors in d_H which are related to T_x in accord with equation (41)

$$\Delta T_x = \Delta\left(\frac{I_{x_1}}{I_{x_0}}\right) = \Delta\left(\frac{d_{H_x}}{d_{H_0}}\right) \tag{41}$$

ΔT_x is influenced by the transmittance of the historadiograph and the uncertainty, Δt_H, in measuring this transmittance. The error in measuring the extinction of a historadiographic structure is at a minimum if its transmittance is 0.38 (Fig. 10). If the historadiographic image is that of a 0.513 μ nitrocellulose foil, then T_x for this foil at 2 kV is 0.81. (Fig. 18). If the transmittance, t_{H_x}, is 0.38, then t_{H_0} can be calculated with equation (36) It then becomes possible to determine $\Delta(d_{H_x}/d_{H_0})$ by using equations (39), (40) and (38) Since $\Delta(d_{H_x}/d_{H_0})$ is equal to ΔT_H, it may be substituted for it in equation (32). It is found that an uncertainty of 0.01 in measuring t_{H_x} gives rise to $\Delta T_x = 0.03$. This uncertainty in measuring the transmittance of a 0.513 μ foil gives rise to a 20% error in its mass determination. It can be stated that a simple value cannot be assigned to the photometric error in historadiography and have it represent the error in mass. One may determine from experimental data the uncertainty in photometry and then calculate the reliability of the mass determination at a given mass range and X-ray tube potential. It is noted in the above calculations that the error in mass for a 0.513 μ foil, resulting from both direct and indirect measurements of I_{x_1} and I_{x_0} is relatively high even with a small photometric uncertainty. This is because of the high transmittance of the sample. Greater accuracy is achieved by direct methods. The error in the indirect method becomes even larger if the transmittance values, t_H, of the historadiograph are above or below 0.38, as is frequently the case.

The relative-transmittance technique

The photographic method of recording X-ray intensities will give rise to a greater error in determining mass than would direct methods if the latter could be used in quantitative historadiography. The high density values generally encountered in photographic methods have definite advantages. This fact becomes evident in the relative transmittance technique and provides a unique method for obtaining the high degree of accuracy required for measuring X-ray intensities in historadiography.

Relative transmittance methods in colorimetry have been discussed by GRIDGEMAN[25] and HISKEY[30]. The principles of the technique are readily adapted to densitometry. In ordinary photometry the blank is placed at 100% transmission and then transmittance values are obtained on standards for the construction of a calibration curve. In the

References p. 321.

relative-transmittance technique, one of the standards is placed at 100% transmission, and the transmittance of the other standards and the unknown are then measured. The standard having transmittance close to that of the unknown is the one selected to be set at 100% transmission. An understanding of the method can be obtained from Fig. 21 and Fig. 23. The method provides a convenient procedure for stretching the scale and thereby increasing the accuracy of the readings.

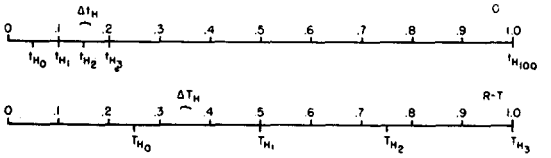

Fig. 21. Illustrating the "scale-stretching" principle in the relative-transmittance procedure.
O – Readings for ordinary photometry; R–T – The same system read using the relative-transmittance principle.

Theory: The absorption of visible light by the historadiograph can be related to the concentration of silver in the film resulting from exposure to X-rays. If C_x and C_0 represent the concentration of silver particles in the image of the reference and clear space (t_{H_0}, Fig. 15) respectively, equations (42) and (43) represent the relationship between concentration of silver particles and the light intensities through the area where the silver is concentrated.

$$d_{H_0} = -\log_{10} t_{H_0} = k \cdot C_0 \tag{42}$$

$$d_{H_x} = -\log_{10} t_{H_x} = k \cdot C_x \tag{43}$$

$$d_{H_0} - d_{H_x} = k(\Delta C)$$

$$\frac{t_{H_0}}{t_{H_x}} = T_{H_0}$$

$$-\log_{10} T_{H_0} = k(\Delta C) \tag{44}$$

HISKEY[30] has shown that the error in measuring concentration by the relative transmittance procedure is given by equation (45)

$$\frac{\Delta C}{C} = \frac{0.434 \cdot \Delta T_H}{T_H [(-\log_{10} T_H) + (-\log_{10} t_H)]} \tag{45}$$

$\Delta C_x/C_x$ and $\Delta C_0/C_0$ are calculated by substituting the appropriate values T_{H_0}, t_{H_0} or T_{H_x}, t_{H_x} in equation (45). $\Delta C/C = \Delta d_H/d_H$ and therefore equation (45) expresses the error in measuring extinctions by the relative-transmittance method.

Equation (46) is the general expression for determining the error in mass for a given photometric uncertainty.

$$\frac{\Delta m_p}{m_p} = \frac{\left[\frac{\left(d_{H_x}\frac{\Delta d_{H_0}}{d_{H_0}}\right)^2 + \left(\Delta d_{H_x}\right)^2}{d_{H_0}^2}\right]^{-1/2} \cdot 0.434}{T_x \log_{10} T_x} \tag{46}$$

While equation (37) represents the uncertainty in measuring the intensity of the X-ray beam by ordinary photometry of the historadiograph, equation (45) represents the uncertainty when the relative-transmittance method is used. Assuming that the "absorption equivalent" of the unknown structure is 0.51 μ, it becomes possible to calculate the expected accuracy of the mass determination when the relative transmittance procedure is used. The transmittance values t_{H_0} and t_{H_x} are the values obtained previously, but must be converted to relative-transmittance values. Taking the special case

References p. 321.

where the "absorption equivalent" is equal to the absorption of the standard wedge that is set at maximum transmittance, then $T_{H_x} = 1.0$, and T_{H_0} is calculated from equation (47).

$$T_{H_0} = \frac{t_{H_0}}{t_{H_x}} \tag{47}$$

T_{H_0} calculated from equation (47) is 0.82. Equation (45) is then used to calculate $\Delta d_{H_x}/d_{H_x}$ by substituting the relative-transmittance, T_H, values. $\Delta(d_{H_x}/d_{H_0})$ is then calculated using equation (38). This value is then substituted for ΔT_x in equation (32) and the uncertainty in d_x is calculated. d_x is directly proportional to mass, and therefore equation (46) is the general formula for calculating error in mass resulting from photometric uncertainty. With the 0.51 μ "absorption equivalent" the photometric error in mass with the relative transmittance procedure is 10%. This value could be reduced by having lower values of t_{H_x} as is indicated in Fig. 22. The curves in Fig. 22 were constructed assuming ΔT_H equal to 0.01, and give the uncertainty in measuring d_{H_x} by the relative-transmittance technique.

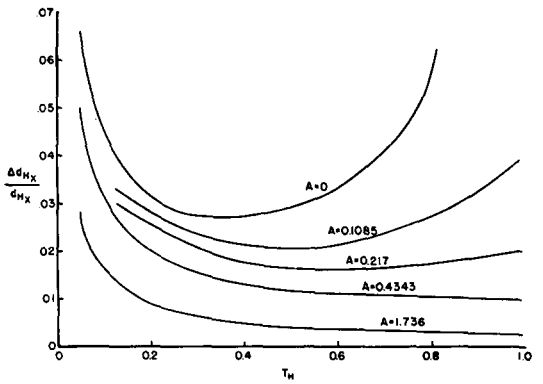

Fig. 22. The error in extinction for values of relative-transmittance.
$A = -\log_{10} t_{H_x}$.

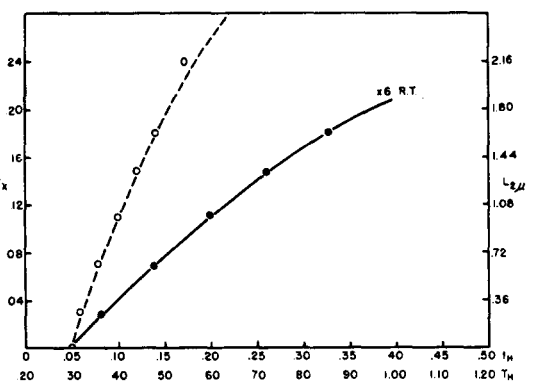

Fig. 23. The theoretical calibration curve.
Curve O – Ordinary photometry;
Curve R–T – Relative-transmittance.

Fig. 23 demonstrates the value of the relative transmittance technique. Curve "O" represents readings by ordinary photometry. Curve "R-T" represents the calibration curve for the relative-transmittance procedure where t_{H_x} was 0.14 and then the sensitivity increased until T_{H_x} was 0.85. This was equivalent to "stretching" the transmittance scale 6 times. ΔT_H may increase, as the sensitivity is necessarily increased to "stretch" the scale. ΔT_H may also increase due to amplifying artifacts in the emulsion. If ΔT_H increases in proportion to the increase in sensitivity there will be no gain in accuracy with the relative-transmittance method. It is obvious that dust-free film, a uniform emulsion, and a stable densitometer are of great importance in achieving high accuracy.

Calibration curves

In establishing calibration curves it is necessary to have an understanding of the values plotted and the expected shape of the curve. It becomes possible to plot a theoretical calibration curve, similar to Fig. 23, by knowing the shape of the intensity-density curve. Fig. 23 was constructed assuming a linear response of the photo emulsion, which

References p. 321.

TABLE I

No.	t_{H_0}	t_{H_x}	$-\log_{10} t_{H_0}$	$-\log_{10} t_{H_x}$	$\frac{-\log_{10} t_{H_x}}{-\log_{10} t_{H_0}}$	d_x	l_2
1	0.05	0.06	1.3	1.22	0.938	0.028	0.25
2	0.05	0.08	1.3	1.10	0.845	0.072	0.65
3	0.05	0.10	1.3	1.00	0.770	0.112	1.02
4	0.05	0.12	1.3	0.92	0.706	0.150	1.35
5	0.05	0.14	1.3	0.85	0.655	0.183	1.65

$$d_x = -\log_{10}\left(\frac{-\log_{10} t_{H_x}}{-\log_{10} t_{H_x}}\right)$$

was justified by the data plotted in Fig. 20. The time of exposure was such that t_{H_0} was 0.05 (Table I). If the structure radiographed does not absorb strongly, the t_{H_x} values for it will be close to 0.05 and therefore t_{H_x} values of 0.06–0.14 were selected. The relationship between these values and d_x is expressed in equation (48).

$$l_2 \sim d_x = -\log_{10} T_x = -\log_{10} \frac{I_x}{I_{x_0}} = \frac{-\log_{10} d_{H_x}}{-\log_{10} d_{H_0}} = -\log_{10}\left(\frac{-\log_{10} t_{H_x}}{-\log_{10} t_{H_0}}\right) \tag{48}$$

Fig. 18 enables a conversion from the d_x values to thickness of the reference foil, which can then be expressed as mass of protein per μ^2. Thus in establishing the theoretical calibration curve $-\log_{10}\left(\frac{-\log_{10} t_{H_x}}{-\log_{10} t_{H_0}}\right)$ is plotted against T_H, whereas in the experimental curve l_2 is plotted against T_H. The curve is practically linear when thin foils (0–1 u) are used.

An estimate of error for the linear portion of the calibration curve is obtained from equation (49)

$$\frac{\Delta l_2}{l_2} = \frac{\Delta T_{H_x}}{T_{H_x} - X} \tag{49}$$

X is the distance from the intercept to zero. ΔT_{H_x} may be taken as the standard deviation when reading the unknown structure. This formula does not take into consideration the uncertainties that may alter the slope of the calibration curve. The value of the R–T method becomes apparent by equation (49) and Fig. 23. If the reference wedge having mass near the unknown is set at $T_H = 1.00$, the value for the unknown will now be near 1.00, and for $\Delta T = 0.02$, the error in mass will be small. Curve "O", Fig. 23 is the calibration curve when the historadiograph is read by setting 100% T through the unexposed film. Curve RT, Fig. 23, is the case if the R–T method is used. Applying equation (49) to these two curves illustrates the advantage of the R–T method. The amplification or "scale stretching" is 6 × for curve "RT", Fig. 23.

Experimental determination of error

Table II and Fig. 24 and 25 present data from an experiment to determine the mass of 0.1–04 μ reference foils by using the historadiographic procedure described in this article. The reference system was made from a 2% Parlodion-dye acetate solution, and the unknown foil from a 1% solution. The thickness of the unknown was determined by the colorimetric method and then compared with data obtained by X-ray absorption (2 kV). The accuracy for this rather ideal system is very good. The deviation noted in the experiment was due primarily to photometric error and the relatively small mass

References p. 321.

TABLE II

EXPERIMENTAL ERROR

No.	l_2	l_{21}*	l_{22}*	l_{23}*	*Average*	*% Error*
B1	0.131	0.180	0.120	0.190	0.163	24.5
31	0.220	0.240	0.200	0.180	0.206	6.3
Eo	0.236	0.250	0.240	0.250	0.246	4.25
D2	0.446	0.475	0.488	0.438	0.454	4.70

l_2 represents values for unknown foils whose thickness was determined by colorimetry. l_{21}*, l_{22}*, l_{23}* are the values from three historadiographic measurements of the same foil.

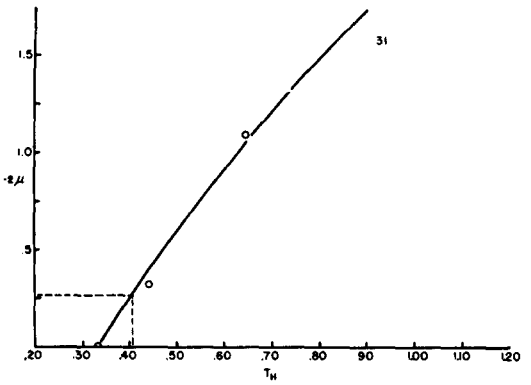

Fig. 24. An experimental calibration curve. The transmittance of the image of the thickest reference wedge was set for maximum transmittance.

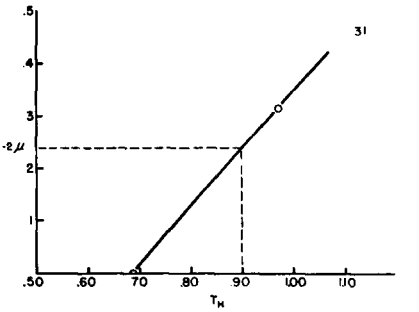

Fig. 25. The same as Fig. 24, but read setting the transmittance of the image of foil closest to the unknown at a maximum.

measured. The error obtained experimentally is in agreement with the theoretical error for the foil. This accuracy cannot always be achieved with the biological samples, as they contain other factors contributing to the error.

The unknown foils were prepared from a different Parlodion-dye-acetate solution than was used for preparing the unknown foil. This indicates a different constant, K_n, and therefore the error expressed in Table II would check the accuracy of the entire historadiographic procedure (Fig. 25).

If the experimental foil had been a 5μ section of tissue, the mass/μ^3 would be 8 g % as calculated with equation (14) or Fig. 2.

DISCUSSION

In defining quantitative historadiographic errors, workers have failed to describe the errors in terms of the quantities involved, the X-ray tube potential, and the transmittance of the material in the X-ray beam. ENGSTROM AND MALMSTROM[31] and LINDSTROM[32] have calculated the random error to be 26%, assuming photometric error of 23%, systematic error in B of 5%, and an 11% error in the reference system. The 23% error in photometry is to be expected for the mass of the tissues (85% water) and the tube potentials (4 kV) used by ENGSTROM[6, 31]. A 5 μ section of tissue originally containing 80% water would require a 0.5 μ reference foil. In Fig. 19 it can be seen that the photometric error in measuring the mass of this foil by direct methods would be 20%

References p. 321.

when the tube potential is 4 kV, but reduced to 7% when the tube potential is 2 kV. Although BRATTGARD *et al.*[33] report reducing the photometric error, it should be noted that they did not reduce the tube potential, but reported the error obtained with tissue having a high protein content ($2.5 \cdot 10^{-9}$ gm/μ^2), amounting to a 50% protein solution, providing the section thickness was 5 μ. These workers[12, 16, 33] have consistently reported the use of thick foils and high protein contents in tissues. In one case[12] the value $0.98 \cdot 10^{-12}$ gm/μ^3 was reported. This would be equivalent to a 98% protein solution. The "absorption equivalent" for the 50% protein tissue sectioned at 5 μ would be 1.2 μ, and the photometric error for this foil at 4 kV would be 3%, assuming $\Delta T_x = 0.01$. The reference system described by ENGSTROM AND LINDSTROM[6, 34] is not as accurate as the one used by BRATTGARD AND HYDEN; however, the accuracy for the reference system reported by these workers was for thick foils (2.8 μ) and the error in their method would be 5–10% for foils of the thickness used by ENGSTROM AND MALMSTROM[31] and ENGSTROM AND GLICK[35]. The increase in accuracy achieved by BRATTGARD *et al.*[33] is more apparent than real, as it was brought about by working at an entirely different mass range and not by improving the technique.

The following factors have been demonstrated to influence the accuracy of the quantitative historadiographic technique and should be considered before undertaking any experiment:

(a) approximate mass of the sample
(b) accuracy of the reference system
(c) tube potential
(d) accuracy of the B value
(e) photometric errors
(f) film inhomogeneity
(g) specimen inhomogeneity
(h) geometric unsharpness.

While the error has been discussed for differences in mass, photometric uncertainty, and tube potential, it should be realized that $\Delta m_p/m_p$ also reflects the minimum amount that can be removed from the tissue to produce a noticeable ΔT_H. This fact is of importance in deciding whether loss in weight resulting from selective solvent or enzyme action can be detected by the historadiographic procedure.

SUMMARY

A thorough discussion of the procedures and errors in quantitative historadiography is presented. Accessory apparatus is described for obtaining accurate values for the X-ray absorption constants used in the calculation of mass. Theoretical considerations are made of the accuracy achieved in measuring X-ray absorption with varying tube potential and mass of the unknown structure. The use of relative transmittance procedures is described for increased accuracy in photometry. Results from an experimental determination of the mass of standards are presented.

RÉSUMÉ

Une discussion approfondie des méthodes et des erreurs en historadiographie quantitative est présentée. Un appareil accessoire qui permet d'obtenir les valeurs exactes des constantes d'absorption des rayons X, utilisées dans le calcul des masses, est décrit. Des considérations théoriques, qui portent sur la précision obtenue dans la mesure de l'absorption des rayons X en fonction de la variation du potentiel de tube et de la masse de la structure inconnue, sont exposées. L'emploi de procédés de transmission relative, qui augmentent la précision en photométrie, est décrit. Les résultats de déterminations expérimentales de la masse de standards sont présentés.

References p. 321.

ZUSAMMENFASSUNG

Methoden und Fehler im Gebiete der quantitativen Historadiographie werden eingehend erörtert. Eine Nebenapparatur zum Erhalten von genauen Werten für X-Strahlenabsorptionskonstanten, die zum Errechnen der Massen gebraucht werden, wird beschrieben. Theoretische Betrachtungen über die Genauigkeit von X-Strahlenabsorptionsmessungen unter Änderung des Röhrenpotentials und der Masse der unbekannten Struktur werden angestellt. Der Gebrauch von relativen Durchlässigkeitsmethoden zum Erhalten von grösserer Exaktheit in der Photometrie wird beschrieben. Ergebnisse experimenteller Bestimmungen von Standardmassen werden gebracht.

REFERENCES

1 P. LAMARQUE, *Bull. histol. appl. physiol.*, 14 (1937) 5.
2 P. LAMARQUE, *Brit. J. Radiol.*, 11 (1938) 425.
3 P. GOBY, *Compt. rend.*, 156 (1913) 686.
4 A. DAUVILLIER, *Compt. rend.*, 190 (1930) 1287.
5 P. LAMARQUE AND J. TURCHINI, *Arch. Soc. sci. méd.*, 18 (1937) 27.
6 A. ENGSTROM AND B. LINDSTROM, *Biochim. Biophys. Acta*, 4 (1950) 351.
7 R. STOWELL, *Stain Technol.*, 26 (1951) 105.
8 B. MALMSTROM AND D. GLICK, *Exp. Cell. Research*, 3 (1952) 125.
9 J. TURCHINI, *Arch. Soc. sci. méd.*, 18 (1937) 113.
10 J. F. RINEHART AND A. SULEIMAN, *Arch. Pathol.*, 51 (1951) 666.
11 R. MELLORS, *Cancer Research*, 13 (1953) 101.
12 S. BRATTGARD, *Acta Radiol., Suppl.*, 96 (1952) 1.
13 G. GLIMSTEDT AND R. HAKENSSON, *Nature*, 167 (1951) 397.
14 P. LANGE AND A. ENGSTROM, *Lab. Invest.*, 3 (1954) 116.
15 A. ENGSTROM AND L. WEGSTEDT, *Acta Radiol.*, 35 (1951) 345.
16 S. BRATTGARD AND H. HYDEN, *Acta Radiol., Suppl.*, 94 (1952) 1.
17 J. J. CLEMMONS AND M. A. APRISON, *Rev. Sci. Instr.*, 24 (1953) 444.
18 V. COSSLETT AND W. NIXON, *Proc. Roy. Soc. (London), B.*, 140 (1952) 422.
19 P. KIRKPATRICK AND A. BAEZ, *J. Opt. Soc. Amer.*, 38 (1948) 76.
20 O. HALLEN AND E. INGELSTAM, *Exp. Cell Research*, 3 (1952) 248.
21 O. HALLEN, *Exp. Cell Research*, 4 (1953) 494.
22 B. LINDSTROM, *Biochim. Biophys. Acta*, 10 (1953) 186.
23 J. J. CLEMMONS AND T. C. WEBSTER, *Biochim. Biophys. Acta*, 11 (1953) 464.
24 F. TWYMAN AND G. F. LOTHIAN, *Proc. Phys. Soc. London*, 45 (1933) 645.
25 N. T. GRIDGEMAN, *Anal. Chem.*, 24 (1952) 445.
26 E. DJURLE AND O. HALLEN, *Exp. Cell Research*, 5 (1953) 301.
27 A. ENGSTROM AND B. LINDSTROM, *Acta Radiol.*, 35 (1951) 33.
28 S. BELLMAN, *Acta Radiol., Suppl.*, 102 (1953) 1.
29 A. POLLISTER, *Lab. Invest.*, 1 (1952) 106.
30 C. F. HISKEY, *Anal. Chem.*, 21 (1949) 1440.
31 A. ENGSTROM AND B. MALMSTROM, *Acta Physiol. Scand.*, 27 (1952) 91.
32 B. LINDSTROM, *Acta Radiol.*, 38 (1952) 355.
33 S. D. BRATTGARD, O. HALLEN AND H. HYDEN, *Acta Radiol.*, 39 (1953) 494.
34 B. LINDSTROM, *Biochim. Biophys. Acta*, 10 (1953) 186.
35 A. ENGSTROM AND D. GLICK, *Science*, 111 (1950) 379.

Received January 17th, 1955