GENERAL CYTOCHEMICAL METHODS

Volume 1

Edited by
J. F. DANIELLI

GENERAL CYTOCHEMICAL METHODS

Volume 1

Edited by

J. F. DANIELLI

Department of Zoology
King's College, London

ACADEMIC PRESS INC., NEW YORK, 1958

PREFACE

This is the first of a series of volumes which will appear at intervals of about two years. Subsequent volumes will each contain a number of descriptions of additional techniques and supplements to previous volumes which will bring the earlier volumes up to date. The endeavor is to include techniques which have been sufficiently studied to eliminate most of the uncertain points which in cytochemistry usually beset a new method.

Ten years ago enough had been done in cytochemistry to show that in this field lay remarkable possibilities of understanding the chemical structure of cells. But most of the methods then available were the subject of sharp controversy. To assist in clarifying the issues involved, the International Society for Cell Biology set up a Cytochemical Commission. The Commission has held a number of round-table discussions which have been of the greatest value. Insofar as agreed common standards of exactness have been achieved, much must be attributed to these meetings. It is hoped that these standards are reflected in this volume.

The first volume of a series such as this is necessarily experimental. The treatment accorded varies in degree of detail in the different articles. It is hoped that readers and users of this volume will let both the authors and the editor know their views on the value and deficiencies of the different manners of treatment. This will be helpful, not only in amending the present volume, but also in designing subsequent volumes.

J. F. DANIELLI

March, 1958
London, England

CONTENTS

Quantitative Determination of DNA in Cells by Feulgen Microspectrophotometry

CECILIE LEUCHTENBERGER

Autoradiography as a Cytochemical Method, with Special Reference to C^{14} and S^{35}

S. R. PELC

The Cytochemical Demonstration and Measurement of Sulfhydryl Groups by Azo-aryl Mercaptide Coupling, with Special Reference to Mercury Orange

H. STANLEY BENNETT AND RUTH M. WATTS

Indigogenic Staining Methods for Esterases

S. J. HOLT

Fluorescent Antibody Methods

ALBERT H. COONS

The Calcium Phosphate Precipitation Method for Alkaline Phosphatase

J. F. DANIELLI

THE WEIGHING OF CELLULAR STRUCTURES BY ULTRASOFT X-RAYS

ARNE ENGSTRÖM AND BO LINDSTRÖM

Department of Medical Physics, Karolinska Institutet, Stockholm, Sweden

I. INTRODUCTION

The aim of modern developments in quantitative histo- and cyto-chemistry is to define the microscopic biological structures in terms of their chemical composition and enzymatic activity, and essentially two

types of technical obstacles have to be mastered in order to analyze chemically as small volumes of biological tissues as one cubic micron (μ^3). The greatest of these difficulties is perhaps the development of analytical techniques sensitive enough to obtain a defined and measurable signal from the very small amounts of substance available for the analysis. Difficulty in obtaining the sample in such a form that it resembles the living tissue as much as possible is the other great troublemaker in quantitative cytochemistry. Naturally the ultimate goal of cytochemistry is to investigate the chemical characteristics of the living cell itself.

In quantitative cytochemistry the unit of weight is 10^{-12} gm. ($= 1\ \mu\mu$g. or pg.) which is the weight of 1 μ^3 of tissue with unit density. As most biological objects contain about three-quarters of water and only one-quarter of dry substance, the weight of the latter portion is about 2.5×10^{-13} gm. in 1 μ^3 of soft tissue. The major part of this dry weight consists of proteinaceous substances, and in many instances components such as fats, carbohydrates, and nucleic acids will *each* not amount to more than a few per cent of the total amount of dry substance. However, in special biological objects the concentrations of the latter compounds may reach high values. Therefore if compounds occurring in concentrations of a few per cent each are to be determined in a volume of tissue of 1 μ^3 with an accuracy of about 10%, the analytical methods must be as sensitive as 10^{-14}–10^{-15} gm.

A considerable number of the methods utilized in quantitative cytochemistry are based on the *absorption* of electromagnetic radiation of various wavelengths ranging from X-rays to ultraviolet, visible, and infrared light. The basis of the absorption methods is given by the fundamental relation between the thickness of the absorbing material present and the attenuation of monochromatic radiation according to the Lambert law,

$$I = I_0 \cdot e^{-\mu l} \tag{1}$$

where I_0 and I are the intensities of the incident and the transmitted radiation, respectively, μ the linear absorption coefficient, and l the thickness of the absorbing substance. Since in cytochemistry very small areas of the sample are to be analyzed, the measurements of the radiation intensities must be performed via an enlarging (microscopic) system.

In addition to the microabsorptiometric methods, techniques based on the *emission* of characteristic radiation (fluorescence) from the sample have found great use. In particular, X-ray microfluorescence procedures have been developed into precise methods for the localization

and the quantitative estimation of minute amounts of trace elements in biological tissues although the most sensitive method for trace metal analysis may be activation analysis in a high intensity neutron beam.

Finally, the diffraction of radiation in biological samples is used to identify chemical components, and especially X-ray microdiffraction and electron diffraction techniques have proven most useful. These methods are of course only applicable to repetitive structures, extensive enough to fulfill the criteria of interference.

In the present communication certain aspects of X-ray microabsorption techniques as applied to cytochemical analysis will be discussed. In particular the determination of the total dry weight and the contents of water of cytological structures *in situ* will be described in detail.

The limit of resolution of present techniques of X-ray microscopy is about the same as that of light microscopy. The least complicated of the X-ray microscopic methods is the direct contact microradiography, generally abbreviated as C.M.R., where the sample, in direct contact with a fine-grained photographic emulsion, is imaged with soft X-rays. Extremely fine-grained photographic emulsions are now commercially available, and the resolution of the contact microradiographic technique is now only limited by the resolving power of the light optical microscope used to inspect the microradiogram. Projection X-ray microscopy and other techniques such as X-ray microscopy based on total reflection, and scanning X-ray microscopy, may, however, give a higher resolution when further developed. However, the very soft X-rays necessary for cytochemical analyses present many technical difficulties which must be overcome before these types of X-ray microscopes can be developed into routine instruments.

TABLE I

TERMINOLOGY OF X-RAYS

Wavelength	Terminology	Main use
< 0.1 Å	Ultrahard	Industrial and medical radiography
0.1–1 Å	Hard	
1–10 Å	Soft	Diffraction, X-ray microscopy
>10 Å	Ultrasoft	

Before discussing the technique of weighing cellular structures by means of X-rays, some definitions must be given regarding the properties of the radiation. The terminology useful for the classification of the wavelength regions utilized for X-ray microscopy is presented in Table I. Mainly, the soft and ultrasoft X-rays must be utilized for high resolution

X-ray microscopy of biological material as is evident from the following discussion.

In the original method for weighing cellular structures by X-rays (Engström and Lindström, 1950) a continuous X-ray spectrum, generated at 3000 volts and filtered through a 9 μ thick aluminum foil, was used. This X-ray spectrum contained wavelengths mainly in the region 8 to 12 A. Recently, however, the technique has been improved to encompass also ultrasoft monochromatic X-rays, in which case the theory of the method can be mastered in an exact way. Moreover, it will be shown in the following section that the wavelengths of the X-rays utilized to determine the mass of biological material can be selected in such a way that a maximum of information can always be obtained from the absorption image of the specimen, whether the biological object has a high or low mass. This is one of the great advantages of the X-ray method in comparison with the microinterferometric mass technique in which the accuracy is determined by the smallest measurable phase shift, thus independent of mass.

II. THE ABSORPTION OF X-RAYS AND THE CONTINUOUS X-RAY SPECTRUM

A. The General Laws for Absorption

In the different wavelength regions of the electromagnetic spectrum the fundamental Lambert law (Eq. 1) is modified in different directions. For X-rays the absorption per unit of mass is usually given instead of the absorption per unit of length, which depends on the physical state of the absorber. If the density of the absorbing element is ρ gm. cm.$^{-3}$, the product $\rho \times l$ represents the mass m of absorbing material per unit of area, expressed in gm. cm.$^{-2}$. Equation 1 then takes the form,

$$I = I_0 \cdot e^{-\frac{\mu}{\rho} m} \tag{2}$$

where μ/ρ is the *mass absorption coefficient* having the dimension cm.^{2} gm.$^{-1}$.

The inner electron shells in the atoms of the absorber are of decisive importance for the absorption coefficient, which is almost completely independent of the physical and chemical state of the absorbing element. It is, however, a function of the frequency of the incident X-rays (wavelength dependence), and of the atomic number of the absorber. A close approximation to the composite mass absorption coefficient, $(\mu/\rho)_{\text{total}}$, of a chemical compound or a mixture, composed of n elements with percentages of weight a_i and individual mass absorption coefficients $(\mu/\rho)_i$, is given by

$$\left(\frac{\mu}{\rho}\right)_{\text{total}} = \sum_{i=1}^{n} \frac{a_i}{100}\left(\frac{\mu}{\rho}\right)_i \qquad (3)$$

The validity of this expression depends on the specific characteristics of the mass absorption coefficients as stated above. Contrary to the case in other wavelength regions, the exact mass absorption coefficients for compounds or mixtures of known composition can therefore be calculated theoretically, once the mass absorption coefficients of the different elements have been determined and tabulated.

For X-rays with wavelengths longer than 0.1 Å passing through matter, the true photoelectric absorption and the scattering are the two processes causing the loss of energy, as expressed by the components of the mass absorption coefficient,

$$\frac{\mu}{\rho} = \frac{\tau}{\rho} + \frac{\sigma}{\rho} \qquad (4)$$

where τ/ρ is the mass photoelectric absorption coefficient and σ/ρ is the mass scattering coefficient. Hence, τ/ρ is a measure of the fraction of incident X-ray quanta, the energy of which is consumed to eject photoelectrons from inner electron orbits of the atoms of the absorber, and σ/ρ is a measure of those X-ray quanta scattered by the electrons of the absorbing material. For X-rays with wavelengths longer than about 2.5 Å, i.e., in the greater part of the soft and in the whole ultrasoft X-ray region, σ/ρ can be neglected in comparison with τ/ρ, and the mass absorption coefficient μ/ρ can then be taken equal to the mass photoelectric absorption coefficient τ/ρ.

The mass photoelectric absorption coefficient is a function of the wavelength λ of the incident X-rays and of the atomic number Z of the absorbing element. An *approximate* empirical formula for this function is given by

$$\frac{\mu}{\rho} = k \cdot \lambda^u \cdot Z^v \qquad (5)$$

where u has a value about three and v a value about four. With given constants the validity of this equation is, however, restricted to a limited range for λ and Z. For each particular element, μ/ρ is a continuously increasing function of the wavelength of the incident X-rays, except for characteristic discontinuities at certain wavelengths. At these critical absorption limits (edges) the total photoelectric absorption in the different orbits of the atoms of the absorber, as expressed by the mass photoelectric absorption coefficient τ/ρ, is greater on the short wave-

length side of this limit than on the long wavelength side, as, on the latter side, no photoelectric absorption occurs in the orbit corresponding to the absorption limit.

B. The Numerical Values of the Mass Absorption Coefficients

When the intensities of incident and transmitted X-rays (I_0 and I in Eq. 2) have been determined experimentally, it is necessary to know

TABLE II

Mass Absorption Coefficients of Elements with Low Atomic Numbers [a]

Absorber	Al Kα 8.34 Å	Cu Lα 13.33 Å	Fe Lα 17.61 Å	Cr Lα 21.57 Å	Ti Lα 27.43 Å	C Kα 44.63 Å
1 H	7.5	30	70	130	260	1000
2 He	30	120	275	500	1000	4300
3 Li	78	280	640	1200	2300	9400
4 Be	152	581	1288	2292	4532	17,430
5 B	324	1233	2711	4784	9200	32,540
6 C	605	2290	4912	8440	15,760	—
7 N	1047	3795	7910	13,120	22,590	3647
8 O	1560	5430	10,740	16,610	1473	5470
9 F	1913	6340	11,600	1015	1949	7280
10 Ne	2763	8240	1079	1863	3575	13,180
11 Na	3129	661	1402	2429	4651	16,650
12 Mg	3797	981	2085	3601	6830	22,850
13 Al	323	1146	2441	4189	7840	24,910
14 Si	510	1813	3812	6420	11,510	33,840
15 P	640	2259	4661	7670	13,280	38,610
16 S	814	2839	5710	9160	15,520	45,230
17 Cl	990	3364	6530	10,210	17,330	50,100
18 A	1163	3795	7110	11,070	18,820	—
19 K	1429	4504	8310	12,960	22,030	—
20 Ca	1706	5150	9450	14,800	24,910	—
21 Sc	1819	5280	9750	15,210	—	—
22 Ti	2002	5680	10,480	16,300	—	—
23 V	2168	6100	11,230	—	—	—
24 Cr	2409	6740	12,360	—	—	—
25 Mn	2556	7160	—	—	—	—
26 Fe	2799	7850	—	—	—	—
27 Co	2956	—	—	—	—	—
28 Ni	3154	—	—	—	—	—
29 Cu	3546	—	—	—	—	—
30 Zn	3685	—	—	—	—	—

[a] From Henke *et al.* (1956).

the magnitude of the mass absorption coefficient μ/ρ in order to calculate the amount m of absorbing material. In the physics literature there are only scattered experimental determinations of the mass absorption coefficients (= the mass photoelectric absorption coefficients) in the soft and ultrasoft X-ray region. However, in a recent publication Henke *et al.* (1956) have devised a semiempirical method for the determination of mass absorption coefficients for X-rays with wavelengths between 5 and 50 Å. A universal function for the absorption by K shell electrons, and another for the absorption by L and extra L shell electrons are given, and these values permit the calculation of mass absorption coefficients for elements with atomic numbers <30. Available experimental and calculated absorption data were compared with those calculated from quantum theory by these authors, and the agreement was very good. Some of the new calculations of μ/ρ for certain important wavelengths in the soft and ultrasoft X-ray regions are presented in Table II.

C. Suitable Wavelength Ranges for Microradiography and the Continuous X-ray Spectrum

Assume a biological sample with the thickness l, where the water has been removed with no change of the dimensions of the specimen as compared with the fresh state. Furthermore, assume that in two adjacent regions there are slightly different total dry weights per unit area, and consequently different linear absorption coefficients μ_1 and μ_2 as the thickness is assumed to be constant. From Eq. 1 the difference between the transmitted intensities, I_1 and I_2 respectively, in the two areas is given by

$$I_2 - I_1 = I_0(e^{-\mu_2 l} - e^{-\mu_1 l}) \tag{6}$$

From this equation the value of l corresponding to the maximum difference between the transmitted intensities can be calculated, and considering a small difference between μ_1 and μ_2, the expression is obtained,

$$l_{max} = \frac{\log_e \frac{\mu_1}{\mu_2}}{\mu_1 - \mu_2} \approx \frac{1}{\mu} \tag{7}$$

where μ is the mean linear absorption coefficient.

In average biological samples in the wavelength range between 5 and 20 Å the value of kZ^v in Eq. 5 is about 1.6. Eliminating the absorption coefficients in Eqs. 5 and 7 the following approximate expression is obtained,

$$\rho \cdot l \approx \frac{1}{1.6 \cdot \lambda^3} \qquad (8)$$

where ρ is the mean of the density of the dehydrated biological sample.

The continuous X-ray spectrum has a sharp short wavelength limit λ_0 determined by the voltage V across the X-ray tube according to

$$\lambda_0 = \frac{12{,}350}{V} \qquad (9)$$

The maximum intensity in the continuous X-ray spectrum is situated at about $3\lambda_0/2$, and then the intensity decreases asymptotically towards longer wavelengths.

Thus, putting $\lambda_0 = 2\lambda/3$, and eliminating λ between the Eqs. 8 and 9, the following approximate expression for V, the suitable voltage across the X-ray tube, is obtained,

$$V \approx 2.2 \cdot 10^4 \cdot \sqrt[3]{\rho l} \qquad (10a)$$

when a continuous X-ray spectrum is utilized. For monochromatic X-rays the corresponding approximate voltage is given by

$$V = 1.5 \cdot 10^4 \cdot \sqrt[3]{\rho l} \qquad (10b)$$

or the suitable wavelength can be obtained immediately from Eq. 8.

For biological specimens of commonly used thicknesses (from 1 to 10 μ) and consisting of between 20 and 35% of dry substance the continuous X-ray spectra must be generated at voltages between 500 and 2000 volts if small mass differences in the specimen shall be visible. Thus, by selecting suitable wavelength ranges (voltages), the X-ray absorption image of a specimen can always be registered under optimum conditions independent of the absolute value of the total dry weight per unit area in the specimen, as already pointed out in the introduction. Figure 1 presents suitable wavelength ranges and voltages for microradiography of soft tissues with varying sample thicknesses and percentages of dry substance.

III. CONDITIONS FOR OPTIMUM, VISUAL, AND PHOTOMETRIC CONTRAST IN X-RAY MICROSCOPIC IMAGES

A. The Definition of a Contrast Function

Before entering the detailed theory of mass determination of cytological structures by X-rays the optimum conditions for contrast in the X-ray microscopic absorption image will be discussed. The presentation will closely follow the contribution by Henke *et al.* (1957). From Eq. 2 it can be seen that the microradiogram "signal" may be described in

terms of a variation in the mass m per unit area or in the mass absorption coefficient μ/ρ, or simply in terms of $(\mu/\rho \cdot m)$, which implies either a change of the mass per unit area or of the chemical composition or of both. The product $(\mu/\rho \cdot m)$ is therefore proposed as the correct number to use as an experimental variable in a microradiographic analysis. This variable may then be "opened up," when reducing the experimental data as allowed or suggested by the experiment itself or by other sources of information.

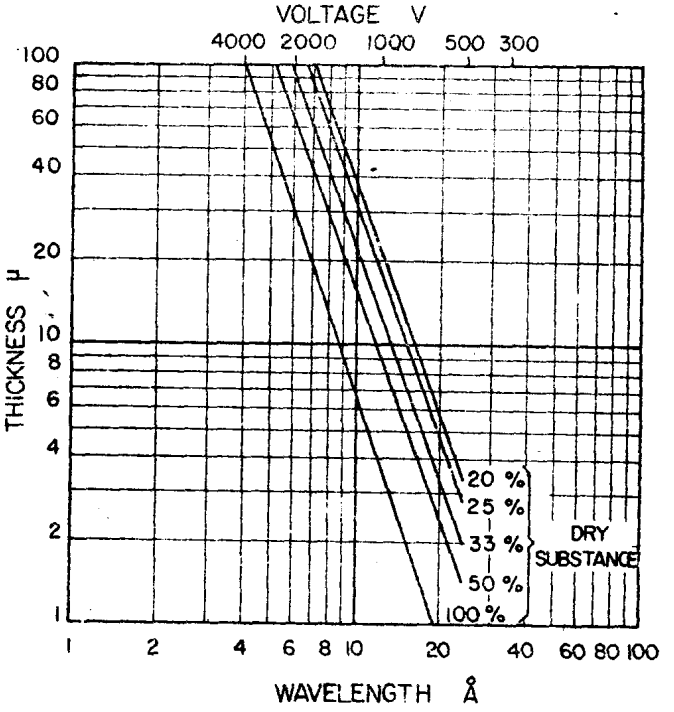

FIG. 1. Suitable X-ray energies for microradiography of soft tissues of varying thickness (Engström, 1955b).

The relative difference between the absorption indices

$$\Delta\left(\frac{\mu}{\rho}m\right)\Big/\left(\frac{\mu}{\rho}m\right),$$

of two specific areas in the specimen is usually the number of primary interest, as the absolute sample thickness is usually of little meaning because of the distortion of the biological structure during preparation, the unreliability of microtome calibrations, and inability to measure accurately the thickness of microscopic sections. The ratio gives relative

variation of the mass or the mass absorption coefficient for the particular radiation—if either the chemistry or the mass per unit area can be assumed constant.

The "contrast" function C is therefore defined by the following relation,

$$C = \frac{-\Delta D}{\Delta\left(\frac{\mu}{\rho} m\right) \Big/ \left(\frac{\mu}{\rho} m\right)} \tag{11}$$

where D is the density in a given region of the microradiogram, defined by the usual relation,

$$D = \log_{10} \frac{i_b}{i} \tag{12}$$

Here i_b and i are the light intensities at the photometric procedure transmitted through an unexposed area and the particular exposed area, respectively, in the microradiogram. Differentiation of Eq. 12 shows that the change in photographic density, ΔD, is proportional to the relative change in the transmitted light, $\Delta i/i$, in a given region of the microradiogram. Therefore ΔD is approximately proportional to the response of the eye to the microradiographic "signal" in this region, and then the "contrast" function is a measure of the visual response per unit relative change of the absorption index. Of course, the eye response is also dependent in a complicated manner on the area associated with the variation of photographic density and on the "distractions" in the neighboring areas of the microradiogram.

B. The Determination of the Contrast Function

The contrast, C, is a function of three variables, ultimately, considered as the wavelength of the X-rays used, the chosen photographic emulsion, and the exposure time. These must be expressed in terms of numbers which can be determined experimentally. The sample X-ray transmission, t, defined as $e^{-\mu/\rho \cdot m}$, can be used to measure the effect of wavelength. The average photographic density in the region of the microradiogram where a variation in density is being evaluated, measures the exposure time for a given sample transmission. Lastly, the density versus exposure data is used to calibrate the chosen photographic emulsion.

In the following discussion the density is expressed as a function of a variable x, proportional to the exposure and therefore also to the exposure time T, as the X-ray intensity is held constant and the reciprocity law is assumed to be valid. Hence,

$$D = f(x) \tag{13}$$

where x is defined by the relation

$$x = k \cdot I_0 \cdot e^{-\frac{\mu}{\rho}m} \cdot T \tag{14}$$

In Eq. 14 I_0 is the radiation flux per unit area at the photographic emulsion with the sample removed, and k is a constant of proportionality.

Differentiating Eq. 14 with respect to $(\mu/\rho \cdot m)$ and recalling that ΔD is equal to Δf according to Eq. 13, $\Delta(\mu/\rho \cdot m)$ and ΔD can be eliminated in Eq. 11. Hence the following expression for C is obtained:

$$C = \frac{\mu}{\rho} m \cdot x \cdot \frac{\Delta f}{\Delta x} \tag{15}$$

The factor $\Delta f/\Delta x$, i.e., the slope at x of the D-vs-x curve, can be determined by numerical differentiation from the D-vs-T table, T being considered numerically equal to x through the choice of the constant k in Eq. 14. This leaves the factor $x(\Delta f/\Delta x)$ in terms of the variable x. By replacing the variable x in Eq. 14 by the corresponding value of D (reading across the D-vs-x table), the table of values expressing the function $x(\Delta f/\Delta x)$ can be obtained as a function of D. Recalling Eqs. 15 and 11, the following relations are obtained:

$$x \frac{\Delta f}{\Delta x} = F(D) = \frac{C}{\frac{\mu}{\rho} m} = \frac{-\Delta D}{\Delta\left(\frac{\mu}{\rho} m\right)} \tag{16}$$

The function $F(D)$ is dependent only upon the emulsion and can be obtained from the D-vs-T calibration data. According to Eq. 16 this convenient function is equal to the contrast per unit absorption index and thus also equal to the contrast for a sample of transmission $1/e$ ($t \approx 37\%$). The contrast function for any other transmission is obtained by multiplying $F(D)$ by the absorption index which is given by $\log_e(1/t)$.

It is interesting to note that the value of $F(D)$, or the contrast per unit absorption index, is also given in terms of the slope of the curve for the density, D, plotted as a function of $\log_{10}T$, (rather than simply T), by the relation,

$$\frac{C}{\frac{\mu}{\rho} m} = 0.434\gamma \tag{17}$$

where the slope number γ is used in analogy to the H and D convention of light photography.