# THE REFERENCE SYSTEM AND ITS INFLUENCE ON THE ACCURACY OF CYTOLOGICAL MASS DETERMINATION BY X-RAYS

by

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The total mass of cytological structures can be determined from measurements of the absorption of soft X-rays in thin tissue sections according to a method reported by ENGSTRÖM AND LINDSTRÖM[1,2]. In this method a microradiogram of a thin biological sample and of a reference system, consisting of a step wedge of nitrocellulose foils, is recorded on a fine-grained photographic emulsion using continuous X-radiation (generated at 3000 volts and filtered through 9 $\mu$ aluminium). In this way it is possible to determine the total mass of structures with areas as small as 5 $\mu^2$. The systematic error does not exceed $\pm$ 5%. The reference system was introduced because of the non-linear relationship between the intensity of the X-radiation and the density of the recorded image (except for very small intensities) and because of the difficulties encountered in calculating an exact composite mass absorption coefficient. The mass absorption coefficient of the reference system, $\left(\frac{\mu}{\varrho}\right)_{CNO_{\mathrm{ref}}}$, must have the same wavelength dependence as that of the tissue, $\left(\frac{\mu}{\varrho}\right)_{CNO_p}$. Neither is corrected for hydrogen.

Equation 10a in the paper by ENGSTRÖM AND LINDSTRÖM[2] gives the mass per unit area of a cytological structure;

$$\frac{m_a}{k_{H_p}} = \frac{k_{H_{\mathrm{ref}}}}{k_{H_p}} \; m_{ref} \; \frac{\left(\frac{\mu}{\varrho}\right)_{CNO_{\mathrm{ref}}}}{\left(\frac{\mu}{\varrho}\right)_{CNO_p}}.$$

$\frac{m_a}{k_{H_p}}$ is the mass of the cytological structure, $k_{H_{\mathrm{ref}}} \cdot \left(\frac{\mu}{\varrho}\right)_{CNO_{\mathrm{ref}}}$ the corrected mass absorption coefficient of the reference system, $k_{H_p} \cdot \left(\frac{\mu}{\varrho}\right)_{CON_p}$ that of the cytological structure, and $m_{\mathrm{ref}}$ the weight of the reference system with the same absorption as the cytological structure under investigation. This last weight is given by $m_{\mathrm{ref}} = u \cdot w$, where $u$ is the foil equivalent of the cytological structure, as determined by comparing the photometer deflection for the structure with the calibration curve of the step wedge (see Fig. 13 in the paper by ENGSTRÖM AND LINDSTRÖM[2]), and $w$ is the weight per unit area of one foil.

The errors in the different factors of this equation have recently been determined by LINDSTRÖM[3]. It was found that the maximum error in the ratio $\frac{k_{H_{\mathrm{ref}}} \cdot \left(\frac{\mu}{\varrho}\right)_{CON_{\mathrm{ref}}}}{k_{H_p} \cdot \left(\frac{\mu}{\varrho}\right)_{CNO_p}}$ could be fixed at $\pm$ 5%. From a series of duplicates the coefficient of variation in $u$ was determined for an average biological tissue (nerve cells). It was $\pm$ 23%. In the same way the coefficient of variation in $w$ was determined ($\pm$ 11%). These values can be combined according to the formula for relative errors of a product; $\pm \sqrt{5^2 + 23^2 + 11^2} = \pm 26\%$. When performing relative measurements the error will be less, for instance $\pm$ 12% when determining the ratio between the masses in the different bands in striated muscles from *Chironomus*. All the figures refer to errors in a single determination. From the above it is clear that the error due to the coefficient of variation in $u$ is most important. This depends on the repeated photographic process, the photometry, the error in the calibration curve, and certain other factors (see LINDSTRÖM[3]).

BRATTGÅRD AND HYDÉN[4] and BRATTGÅRD[5] claim quite another accuracy for the method (a random error of about $\pm$ 3.5%), but they use figures obtained from the literature[6,7,8] when evaluating the error in the photographic-photometric procedure. These figures refer to different equipment, different photographic emulsions and different wavelength regions, and *they are therefore by no means representative of the X-ray weighing procedure.* This is the reason why LINDSTRÖM gets $\pm$ 23% and BRATTGÅRD AND HYDÉN about $\pm$ 3.5% for the same biological material (nerve cells). The figure $\pm$ 23% is an upper limit, and certain improvements (direct photometry in the micro-

radiogram, which ENGSTRÖM[9] was the first to use for biological material) will give a smaller value. For particularly suitable material it is possible to get a still better accuracy with the photographic-photometric procedure (see ENGSTRÖM AND LÜTHY[10]), but in the author's opinion a figure of about ± 10% will be the lowest obtainable. *The only permissible way of determining the error is to investigate a series of duplicates* (or determinations, equivalent to duplicates) *and to use the exact procedure for which the error is being determined.* Differences between materials make it necessary to determine the error for every particular investigation if reliable figures are to be obtained.

The foils for the reference system have been made in the following way by ENGSTRÖM AND LINDSTRÖM: About 10 to 20 drops of a diluted zapon varnish solution are floated on the surface of distilled water in a clean glass dish, the diameter of which is 8–9 cm. In the bottom is a metal ring with a diameter of about 6 cm. When the zapon varnish begins to wrinkle on the edges, the metal ring is raised out of the water. The zapon foil, which is suspended in the metal ring, is then dried in a dust-free chamber. A certain area of each foil is cut out and weighed on a precision microbalance (with a maximum error of ± 0.005 mg). The weighed part of the foil and the adjacent parts are used as reference systems. In most experiments the weight of the foil, $w$, has been about 0.2 mg cm$^{-2}$.

BRATTGÅRD AND HALLÉN[11] obtain an error of ± 18–20% for this procedure, but in a series of duplicates LINDSTRÖM[3] reports ± 11%. The variation could be due to certain differences in the technique used and in the precision of the balance. "The earlier method b" in the paper by BRATTGÅRD AND HALLÉN[11] must be their own invention, for it has never been used by ENGSTRÖM AND LINDSTRÖM. BRATTGÅRD AND HALLÉN[11] describe an improved method in which, for weights less than 0.1 mg cm$^{-2}$, the error is ± 6%, but for weights over 0.2 mg cm$^{-2}$ the error is ± 10–14%. This is about the same error as given by LINDSTRÖM[3] for the earlier method (± 11%). It is true that this early method is not good for making very thin foils. On the other hand these thin foils (0.1 mg cm$^{-2}$) are difficult to handle when putting the step wedge very close to the biological structure to be measured.

A modification of the preparation holder has been introduced by BRATTGÅRD AND HYDÉN[4]. The holder is made in two parts, one for the reference system and the other for the biological sample. With this procedure the same reference system can be used in many experiments. In this case the weight of the supporting membrane of the sample must be determined for every preparate with an inevitable new error.

The most important improvement in the reference system is the interferometric determination of the thickness of the foils (HALLÉN AND INGELSTAM[12]). In their paper the precision was given as a few per cent for a Young double slit system, but BRATTGÅRD AND HYDÉN[4] claim 1%. From an accurate value for the specific gravity of the foil, the mass per unit area of one foil, $w$, can be determined. The interferometric procedure is also used when checking the uniformity of the foils. In the original method[2], uniform photometric values over a whole step in the wedge give the necessary information about the evenness of the foil.

The improvements in the determination of $w$ are of little value when relative measurements are used, for then only values of $u$ are considered. As the error in $u$ (*i.e.* the error in the photographic-photometric procedure in X-ray microradiography) is the most important one, an isolated reduction of the error in $w$ has a rather small influence upon the overall error in absolute measurements. When an electrical detecting system can be used instead of the photographic-photometric procedure the accuracy will increase considerably, and in this case the reference system will not be necessary.

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