A CRITICAL EVALUATION OF X-RAY MICRORADIOGRAPHY

by

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Recently, an article by LINDSTRÖM[21] discussing the accuracy of cytological mass determination by X-rays appeared in this Journal. On the basis of the results obtained by ENGSTRÖM AND LINDSTRÖM[11,12] the authors criticise a series of papers published from the Histological Department of the Medical School in Göteborg[2-7,10,16,18]. These papers deal with the errors of X-ray microradiography and the possibility of their elimination, describing new constructions and procedures for this purpose.

LINDSTRÖM[20,21] calculates his over-all random error to be $\pm$ 26%. According to our results, using the procedure described by ENGSTRÖM AND LINDSTRÖM[12], this value is too low. It probably amounts to 50–70%. ENGSTRÖM AND LINDSTRÖM have not taken into consideration certain important errors which can be reduced or eliminated. LINDSTRÖM discusses some sources of error of which the three main errors are:

1. the error in the photographic-photometric procedure;
2. the error in the reference system;
3. systematic and theoretical errors.

Three other sources of error must be considered when the method is used for the determination of mass and for the analysis of chemical fractions per volume of biological material. These are:

4. errors in the determination of the volume;
5. errors due to the biological material and to the fixation;
6. errors in the extraction and digestion of the material.

1. In *the photographic-photometric procedure* used by ENGSTRÖM AND LINDSTRÖM "the microradiogram is enlarged by photomicrography"[20]. They found the error of a single determination to be 23%. The magnitude of this error does not agree with the experience of density measurements on plates[8,9]. This large error cannot be mainly due to the photometric procedure. We have shown that it is mainly due to the secondary enlargement of the radiogram. In a series of measurements exposing the same reference system eight times on the same plate with stabilised illumination and constant exposure time, we found the standard deviation to be $\pm$ 36% (3σ = 108%!). Therefore we have abandoned the use of secondary enlargement.

We use instead a specially constructed microphotometer with linear amplification to measure the density directly in the primary radiogram[2,3,7]. The random error in our photographic-photometric procedure for the X-ray determinations was experimentally determined to be $\pm$ 1.5%. This figure agrees well with those given in the literature[17,19], and is not "obtained from the literature"[21]. Thus we have considerably reduced the large error of 23% stated by LINDSTRÖM[20,21].

2. *The error in the reference system.* LINDSTRÖM calculates the error to be $\pm$ 11% based on *21 determinations*[20]. Our investigation[6] comprises *1400 determinations*. These show clearly that the weight of *two adjacent parts* of the foil made according to ENGSTRÖM AND LINDSTRÖM[21] can vary up to 55%. Using a special apparatus for the preparation of these films[3,6,7], we found that the corresponding variation was 18%. This error was so large that we had to abandon the gravimetric method. The fact is that *every cellulose film* produced on a water surface, even if it is made with the greatest care, varies considerably in thickness in adjacent parts. We have repeatedly emphasised[3,7,18] that the same part of the reference system that is used for the absorption measurements must also be measured with respect to mass. A gravimetric method requires a surface of at least 1 cm^2. For the X-ray absorption measurements we use a surface of the film of 0.2 mm^2 constituting each step of the reference system. In the same area the mass of the reference system is now determined interferometrically according to HALLÉN AND INGELSTAM[16] and DJURLE AND HALLÉN[10]. This method has an accuracy of $< \pm$ 1%. It is quite true that it is of little value to reduce the error in the reference system (in LINDSTRÖM's case $\pm$ 11%) if the photographic-photometric error amounts to 23%. But as soon as this latter error is reduced, as we have done to $\pm$ 1.5%, the accuracy of the method depends upon the reference system. Therefore, the two errors must be reduced to the same level.

3. *Theoretically calculated errors.* The X-ray method is based on the fact that the carbon, nitrogen and oxygen in the tissue account for the main part of the absorption of soft X-radiation[11,12]. These elements taken in the same proportions as in proteins are assumed to be responsible for the total absorption. A correction is made for the presence of hydrogen. However, other elements than C, N, O and H contribute to the total absorption. The presence of these other elements[3,7], therefore, gives a *positive systematic error* $\leqq$ 5%.

This value is erroneously used as a random error by ENGSTRÖM AND LINDSTRÖM[14,20,21] in their calculation of the error of the method. It is clear that the distribution of these other elements in a cell may vary, and so give rise to a random error, but this error belongs to the total random error of the *biological material*.

4. *Errors due to mass determination per volume.* The X-ray method gives information about mass per unit surface. If the thickness of the section is known, the mass per unit volume can be determined. In determining the thickness by optical measurements two errors must be considered. One is a random error theoretically calculated to be $\pm$ 2% at a thickness of 10 μ[1,3]. This figure is experimentally confirmed. The second error is systematic and due to changes in the fresh tissue caused by fixation, sectioning, treatment with chemical substances and vacuum. If possible this error must be determined from case to case. In some of our experiments these factors have caused the preparation to shrink to half the original thickness of the fresh tissue. This may partially explain also some high dry weight values ($0.7-1.1 \cdot 10^{-9}$ mg/μ^3) of lipid-extracted cell material published by NÜRNBERGER, ENGSTRÖM AND LINDSTRÖM[22].

It is to be noted that in our papers the mass is expressed per optically determined volume of the treated and structurally changed material. *These values must not be referred to volume of fresh tissue.*

5. *Errors due to variation in the biological material.* These errors must be determined experimentally with due regard paid to sampling. In an investigation by NÜRNBERGER, ENGSTRÖM AND LINDSTRÖM[22] on fixed anterior horn cells they found a large standard deviation (σ) of $\pm$ 30–45 % BRATTGÅRD[4,5] and GOMIRATO[15] showed that these cells can be divided in two statistically significant cell groups, one with a high and the other with a small mass. This fact has escaped NÜRNBERGER, ENGSTRÖM AND LINDSTRÖM[22] but explains their large sigma values. A failure of this kind to detect two significant groups of cells exemplifies the danger in using a method the large experimental error of which encompasses biologically significant data.

6. ENGSTRÖM AND LINDSTRÖM have suggested[12] that the method be used for the determination of chemical fractions by extraction and digestion of cells.

We have published a detailed method for a procedure of this kind for nerve cells[3,7,18]. In these papers we stress the necessity of concomitant chemical controls, and of determining the systematic error. LINDSTRÖM cites[21] the study of ENGSTRÖM AND LÜTHY[13] in which lipids were determined by extracting nerve tissue for 72 hours with alcohol-ether and petroleum ether. But to do this and not check whether the treatment extracts other X-ray absorbing substances is not permissible. Moreover, these authors publish values obtained from structures the magnitude of which (1.2 μ) is below the resolution of the LIPPMANN film[7].

X-ray microradiography originally elaborated by ENGSTRÖM AND LINDSTRÖM is an excellent contribution to the arsenal of the cytochemical methods.

But like all new methods it needs improvement, and the first step is to reduce or eliminate all the obvious sources of error.

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