X-RAY MICRORADIOGRAPHY AS A QUANTITATIVE METHOD

by

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THE REFERENCE SYSTEM

In 1949–50, ENGSTRÖM AND LINDSTRÖM[1,2,3,4] reported a method for the determination of the mass of extremely small biological objects. The method is based on the fact that the absorption of a certain continuous X-radiation—calculated for C, N, O and H in the proportions in which they are present in the cell—may be considered as directly proportional to the mass. This applies if the wave-length is around 8 A. It is not possible to make measurements of the transmission of X-radiation through a cell. A comparison is therefore made with the transmission in a reference system composed of nitrocellulose films of uniform thickness, arranged in step and of known composition and mass per unit of surface.

Using equation

$$\frac{m_a}{k_{\mathrm{Hp}}} = \frac{k_{\mathrm{H\,ref}}}{k_{\mathrm{Hp}}} \cdot m_{\mathrm{ref}} \cdot \frac{\left(\frac{\mu}{\varrho}\right)\mathrm{CNOref}}{\left(\frac{\mu}{\varrho}\right)\mathrm{CNOp}}$$

where m_a/k_{Hp} is the weight of the biological structure under investigation, m_{ref} the weight of the reference system with the same absorption, $k_{\mathrm{H\,ref}}$ is the connection factor for the hydrogen in the reference system, k_{Hp} in the biological structure, (μ/ϱ) CNO ref is the mass absorption coefficient of the reference system and (μ/ϱ) CNOp is the mass-absorption coefficient of the biological structure (10 *a* in ENGSTRÖM AND LINDSTRÖM'S work[4]) we get

$$m = \mathrm{const.} \cdot g \cdot f$$

where g is the weight of the reference system per unit of surface and per unit of film and f is the thickness of a reference system with the same absorption as that of the relevant preparation. The mass in question may then be calculated per unit of surface, $m \cdot f$ being expressed in units of folio, *i.e.*, the number of the steps in the reference system. It is evident from the formula that the accuracy of the method is essentially dependent on the characteristics of the reference system. These characteristics will be dominated by two factors, *i.e.*,

1. The composition of the nitrocellulose.
2. The quality of the nitrocellulose film.

The first of these factors has been thoroughly analysed by ENGSTRÖM AND LINDSTRÖM[4].

For the nitrocellulose film to be good quality, the mass must be evenly distributed throughout it. Moreover, the thickness of the foil must be such that the absorption of the different steps in the reference system will cover the range of the absorption in the cell. In our investigations, a foil 0.5–8 μ thick was found to be suitable.

EARLIER METHODS

When preparing nitrocellulose films of the aforementioned thickness, a solution of cellulose varnish is allowed to spread on a surface of water in an open vessel. When a film has formed, one of the two following methods, differing in principle, may be chosen:

a. The foil is lifted up with a metal ring or cylinder and left to dry.

b. The foil is lifted into a shiny paper after evaporation of the solvent.

a. *The foil is lifted up with a metal ring or cylinder* and allowed to dry on it. Pieces of the film obtained in this way are then torn off and used for the reference system. Adjacent pieces are used for weighing, after determination of their surface. Weight analyses have been made in order to obtain a check on the reference system prepared with this method. Pieces with an area of 4 cm^2 were punched out with a smaller cylinder from the nitrocellulose film which was lifted up and allowed to dry on a large brass cylinder. These pieces were taken from up to six different parts of the film, the manipulations took place under a dissection microscope. The pieces removed were weighed, and calculations made of the variations within the surface of the same film. Fig. 1 shows this variation, expressed in σ as a percentage of the mean value, for foils of different thickness. The variations amounted to 18.2% (σ as a percentage of the mean value). (For the statistical formula see BRATTGÅRD *et al.*[17]) The reason for the appreciable variations in weight is presumably to be sought in the considerable differences in tension which arise on taking up the film and during the drying process. If the thickness of the foils is less than that used for the controls, small, scattered holes are seen under the microscope. This is a strong indication that the method is unsuitable for nitrocellulose films with a weight below about 8–10^{-2} mg/cm^2.

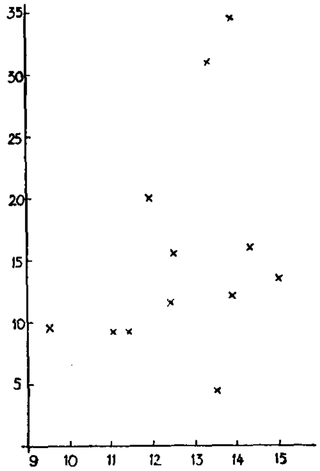

Fig. 1. Variations in weight of films of different thicknesses prepared by the earlier method Ordinate: the dispersion, σ, a. Nitrocellulose: dilution 7:3. stated as a percentage of the mean of the film. Abscissa: the weight in mg·10^{-2} per cm^2

b. *The foil is lifted onto a shiny paper after evaporation of the solvent.* Pieces of the foil of suitable size are then torn off and used for the reference system. The adjacent areas of the nitrocellulose film are cut out together with the paper from which the film is removed, and weighed, the paper being subjected to planimetry.

Weight analyses revealed appreciable differences in various parts of the same foil. A series of investigations, covering approximately 400 samples, showed considerable variations, as may be deduced from Fig. 2. Expressed in σ as a percentage of the mean value, the variation amounted to 20% (see Fig. 2). The analyses also showed that the difference in the weight of two adjacent parts of the same nitrocellulose film could be as large as the difference in weight of its central and peripheral parts. The surface of the paper on which the foil was removed from the solution was found to play a considerable role. Even the finest possible paper surface—for example, chalked cardboard or satin cardboard—showed considerable irregularities under the microscope. Since, when drying, the film takes the shape of the mount, this causes the surface on the film to be uneven. This is illustrated by Fig. 5. Another important consideration is that the aforementioned method is not suitable for films with a weight below $10 \cdot 10^{-2}$, since such films cannot be detached completely from the paper. The variations in the weight of films produced with both methods a and b are nevertheless so large that they prejudice the microradiographic results.

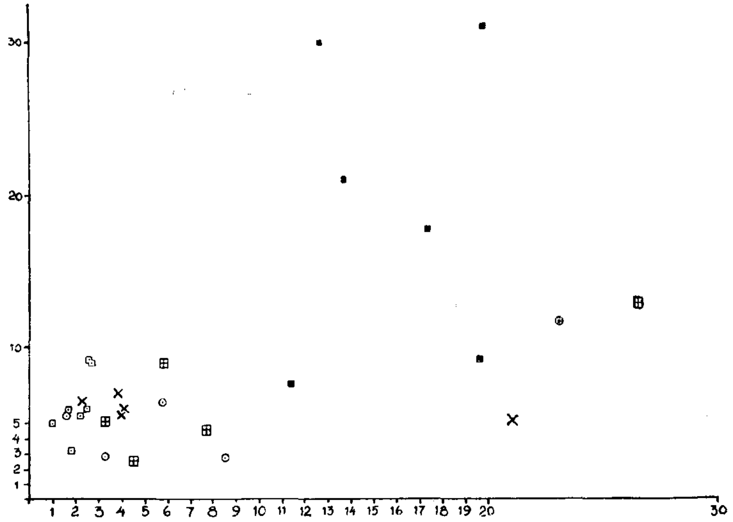

Fig. 2. Variations in weight of films of different thicknesses, prepared with varying dilutions of nitrocellulose and by two different methods. Ordinate: the dispersion, σ, stated as a percentage of the mean of the film. Abscissa: the weight in mg $\cdot 10^{-2}$ per cm^2.

■ = The earlier method. b. Nitrocellulose: dilution 7:3
⊞ = The new method. Concentrated nitrocellulose
◉ = The new method. Nitrocellulose: dilution 1:1
× = The new method. Nitrocellulose: dilution 1:2
⊡ = The new method. Nitrocellulose: dilution 1:3

THE PROBLEM

The aforementioned investigation shows that considerable deficiencies are inherent in the earlier methods for the preparation of a reference system. This implies that the quantitative microradiographic analyses of the mass will be unreliable. We have therefore considered it warranted to discuss those factors that are essential from a physicochemical aspect for the formation of 2–8 μ thick surface films produced by means of spreading of cellulose varnish on a surface of water. On the basis of these theoretical aspects and of our previous experience, we have elaborated another method. It involves fewer sources of error in the reference system and the manualar procedure is the simplest possible.

THEORETICAL ASPECTS

LANGMUIR[5–7] has made a thorough study o the physicochemical problems involved in the spreading of fluid on a surface of water. BLODGETT AND LANGMUIR[8] have described a method (the dip-method) for the preparation of multilayers which, on practical grounds, is inapplicable to our work. Investigations with particular reference to cellulose films have been made by BARTON, HUNT[9], KATZ AND SAMWELL[10–11], HARDING AND ADAM[12] and by WALL AND ZELIKOFF[13]. All these studies, however, deal with monolayers. WYCKOFF[14], among others, has described a procedure for the preparation of cellulose films in connexion with their use as supporting membranes in electron microscopy. He does not deal with the quality of the film with regard to the distribution of the mass. The thickness of supporting membranes of this type is usually from 50 to 150 A (RÅNBY[15]).

It is not possible to compare the formation of a 2–8 μ thick nitrocellulose film on water with that of a monomolecular film. In the former case, the thickness corresponds to more than 1000 molecules. This implies an extremely complicated physicochemical process, particularly with regard to the evaporation of the solvent. It is only possible to establish the following essential factors:

1. *Surface tension.* The spreading of a cellulose solution on a surface of water continues until an equilibrium has been reached between the sum of both surface tensions: water against cellulose solution and cellulose solution against air and the surface tension of water against air, respectively.

2. *Temperature of the water.* Variations in the temperature of the water are of minor importance for the surface tension. However, if the temperature is too low, condensation phenomena occur which cause a milky opalescence of the film. The temperature of the water should not, therefore, differ appreciably from that of the surroundings.

3. *Purity of the water.* Fine particles of dust on the surface of the water have a strong surface action and thus disturb the course of spreading of the cellulose varnish.

4. *Viscosity.* The higher the viscosity of the cellulose solution, the more slowly does the spreading take place. The lower the viscosity, the greater is the volume of the solution that must be spread in order to obtain a film of a certain thickness. It is therefore necessary to avoid extremes.

5. *Evaporation.* During the process of spreading, the air above the surface of the water should be saturated with solvent. This inhibits any tendency of the cellulose solution to enter a solid phase in the course of spreading, which would otherwise be a disturbing factor.

6. *Creasing tendency of the film.* Two essential changes presumably occur when the solvent evaporates. One is the striving of the molecular structure to attain a new state of equilibrium. The other is a change, in particular, of the interfacial surface tension, under the condition of equilibrium enumerated in point 1. Both these factors endeavour to decrease the surface of the film; this results in a tendency to crease, which always constitutes a serious risk of variations in the thickness. The formation of creases is symmetrical and varies within the different radial zones. It is therefore important for all that part of the nitrocellulose film that is to be used to be taken from the same radial zone. This zone should show a minimal tendency to crease.

7. *Manipulation.* During the drying process, manipulation of the film should be avoided. Our investigations have shown that uneven stretching results in considerable variations in the tension and thickness of the nitrocellulose film.

8. *Interference phenomena.* Sometimes interference phenomena occur in the films. Usually the zones show a transitional spectral range but well defined boundaries sometimes appear. These phenomena are due to variations in film thickness. Measurements with a MICHELSON interferometer show that these variations may amount to 0.6 μ. Films showing such interference phenomena must be discarded.

METHOD AND APPARATUS

On the basis of the considerations discussed in the foregoing, we have worked out a method for the preparation of nitrocellulose films. It permits conditions that are, as far as possible, ideal for their production and simplifies the application of the reference system.

The apparatus (see Figs. 3 and 4) consists of a circular vessel (A) in which a supporting plate (B) is inserted. This plate is pierced with 8 symmetrically spaced holes, from which project cylinders (C) with an apical surface of 4 cm^2. Saturated solvent vapour can be sprayed into the cylinders from below through fine cannulas (G).

The vessel is filled with water of suitable temperature through the lower hole (D in Fig. 4) so that the surface of the water is above the level of the supporting plate (B). Solvent vapour is then sprayed through (E) to bring about saturation above the surface of the water. The cork (F) is withdrawn and the desired quantity of nitrocellulose varnish is allowed to run out of a pipette, which is held perpendicularly in the centre, with the tip immediately above the surface of the water.

The film is allowed to dry until slight creases appear in its periphery. It is then lowered onto the supporting plate (B) by slowly draining off water through the bottom hole (D). When the film impinges on the supporting plate (B) and the cylinders (C), saturated solvent vapour is sprayed through (G). This keeps the film in the cylinders flat and prevents it from bulging downwards as the level of the water falls. When the water reaches the grooves in the cylinders, it is regulated automatically. It can then be drained off rapidly and a hot-air fan connected to the bottom hole (D). It is possible with this procedure to avoid any manipulation of the wet film.

When the film is dry, it is cut out around the cylinders (C) in the space between them and the supporting plate (B). The cylinders are lifted out and all the film material outside their top opening carefully scraped off under a magnifying lens. The desired number of foils are used for the reference system and the remainder removed from the respective cylinders under a microscope. These pieces are then rolled into small pellets, dried to a constant weight and micro-weighed.

RESULTS OF THE NEW METHOD

The investigation is based on more than 800 determinations with our method. Eight separate parts of each nitrocellulose film—*i.e.*, those contained in the cylinders—were weighed.

Fig. 2 shows the results of the analyses of the variations in weight of films of different thicknesses and prepared from varying dilutions of nitrocellulose. The dispersion, σ, is stated as a percentage of the mean figure for the relevant foil. It is apparent from Fig. 2 that, with a weight of less than $10 \cdot 10^{-2}$ mg/cm^2, the dispersion lies around

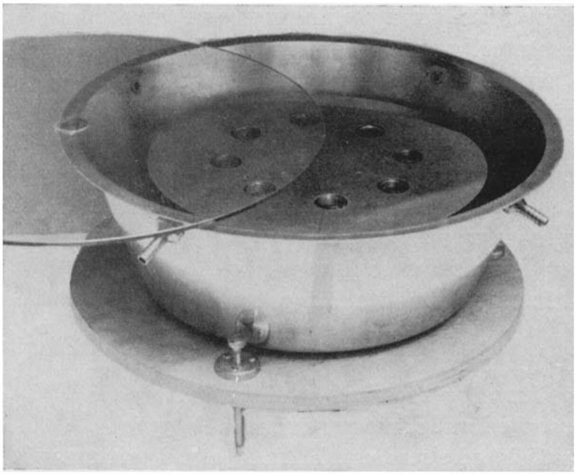

Fig. 3a. The complete apparatus

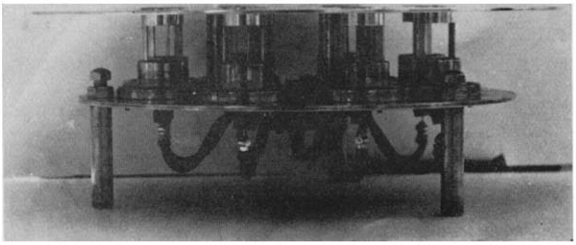

Fig. 3b. The supporting plate removed from the circular vessel

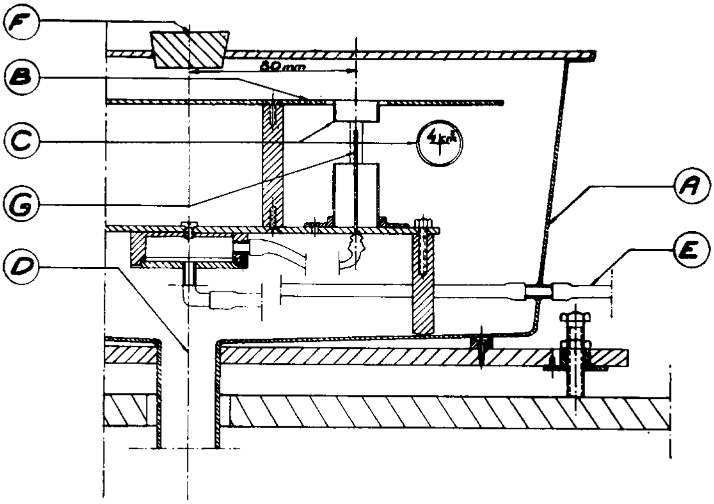

Fig. 4. Schematic diagram of the apparatus: A. Circular vessel; B. Supporting plate; C. Cylinder; D. Bottom hole; E. Solvent vapour line; F. Removable cork; G. Cannulas

6%. The corresponding figure for a thickness of over $20 \cdot 10^{-2}$ mg/cm^2 is 10–14%. It is therefore preferable to use films with a thickness of less than $10 \cdot 10^{-2}$ mg/cm^2. Moreover, nitrocellulose films of this thickness show an absorption that is in good agreement with that of the cell material (nerve cells) that we have hitherto studied. It is also evident from Fig. 2 that variations in the dilution of the cellulose solution do not appear to have any great effect on the dispersion. The evenness of the foil is evident from Fig. 5, in which the right part represents a nitrocellulose film prepared according to our method, the left part showing a film prepared with the earlier method.

Fig. 5. The borderline between films prepared by the earlier method (left) and the new method (right)

Interferometric determinations of cellulose films have shown that films produced by the method of the authors are of uniform thickness, the limit of measurements being 300 A. This results will be published elsewhere (Hallén and Ingelstam[16]).

Comparison between the methods

A comparison was made between our method and those used earlier. It was based on over 800 determinations of the weight in the former case and 600 the two latter. The results may be summarized as follows:

1. The use of the earlier methods is confined to nitrocellulose films of a certain minimal thickness. Our method has no such limitation and also gave distinctly more reliable results with thick films (see Figs. 1 and 2).

2. Weight analyses of the nitrocellulose films prepared with the earlier methods showed an error, expressed in σ as a percentage of the mean figure, of around 18–20%. The corresponding figure with our method was approximately 6% (see Fig. 2).

3. Fig. 5 shows the difference in the evenness of nitrocellulose films prepared with the old and the new method, respectively. The photograph was prepared by producing one half of the nitrocellulose film lifted onto a paper and the other half with our method. Expressed in σ as a percentage of the mean figure, the error of weight in the two halves was 18 and 10%, respectively.

SUMMARY

An account is given of a method for the preparation of thin nitrocellulose films. They are intended for use as a reference system in the determination of the mass of extremely small biological objects according to Engström and Lindström's microradiographic method.

The factors affecting the formation of a nitrocellulose film of the desired thickness are discussed. An apparatus simplifying their preparation is described.

A comparison is made between nitrocellulose films produced with the earlier method and with that suggested by the authors. It shows that the variations in weight amount to 18–20% in the former cases and to 6% in the latter.

RÉSUMÉ

Les auteurs décrivent une nouvelle méthode de préparation de films de nitrocellulose minces. Ces films sont destinés à servir de système de référence dans la détermination de la masse d'objets biologiques extrêmement petits d'après la méthode microradiographique de Engström et Lindström.

Les facteurs qui influencent la formation d'un film de nitrocellulose d'épaisseur désirée sont discutés et un appareil qui simplifie leur préparation est décrit.

Les films de nitrocellulose préparés d'après les méthodes anciennes et d'après la nouvelle méthode sont comparés. Il appert que les variations de poids sont de 18–20% dans les premiers cas et de 6% dans le dernier cas.

ZUSAMMENFASSUNG

Eine Methode zur Herstellung dünner Nitrozellulosefilme wird beschrieben. Sie sollen als Referenzsystem für die Bestimmung der Masse von ausserordentlich kleinen biologischen Objekten nach der ENGSTRÖM-LINDSTRÖM'schen mikroradiographischen Methode dienen.

Die Faktoren, welche auf die Bildung von Nitrozellulose-Filmen von gewünschter Dicke von Einfluss sind, werden erörtert. Ein Apparat zur vereinfachten Darstellung dieser Filme wird beschrieben. Nitrozellulosefilme, welche nach älteren und nach der neuen Methode hergestellt worden sind, werden verglichen.

Im ersten Falle wurden Gewichtsunterschiede von 18–20 %, im zweiten nur von 6 % beobachtet.

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