QUANTITATIVE CYTOCHEMICAL DETERMINATION OF NITROGEN BY X-RAY ABSORPTION SPECTROGRAPHY

I. THE THEORETICAL BASIS

by

A. ENGSTRÖM

Department for Cell Research, Karolinska Institutet, Stockholm (Sweden)

INTRODUCTION

To be able to analyse and follow the intracellular changes and metabolism of nitrogen, one must have the possibility of recourse to methods whose sensitiveness is far greater than the sensitiveness of the ordinary microchemical analysis procedures. If the object is to determine the nitrogen within a cube with an edge of 10 μ of a normal biological tissue, i.e., within the volume which corresponds to the size of an ordinary mammalian cell, the amount of nitrogen which is to be determined will be in the order of magnitude 10^{-11} g, if the average nitrogen content is 3 %.

By means of X-ray absorption spectrography it is possible to determine very small amounts of different elements, as was shown by ENGSTRÖM[1,2], ENGSTRÖM and LINDSTRÖM[3]. In these publications the principles for X-ray spectrographic ultra-micro analysis are laid down, and a number of experimental results with determinations of elements with higher atomic numbers than 15 are presented.

The technical difficulties connected with X-ray absorption analysis increase greatly, however, with falling atomic numbers, owing to the fact that the radiation employed must have a very long wavelength. For 15 P, for instance, if the K-absorption edge is used for the analysis, radiation of a wavelength of c. 6 Å is required, corresponding to a voltage of about 2000 V, while in the case of nitrogen determination the radiation used must have a wavelength of 31.1 Å (c. 400 V) if the K-absorption edge of the nitrogen is to be used. This latter extremely long wave radiation is very greatly absorbed by air, and therefore the whole analysis must be carried out in a high vacuum.

CALCULATION OF THE POSSIBILITIES FOR ANALYSIS

In the case of nitrogen determinations by X-ray absorption spectrography, the absorption of monochromatic X-rays with wavelengths lying on either side of the K-absorption edge for nitrogen is measured. For the following calculations the specimen is assumed to be made up of a nitrogen component X, and a component (q—X) comprising the other elements in the specimen. For the calculations it is necessary to know the magnitude of the mass absorption coefficient for nitrogen within the wavelength range of the K-absorption edge. If the two wavelengths employed for measuring lie quite close to each other and on either side of the absorption edge it is not necessary, as appears

References p. 433.

below, to know the magnitude of the mass absorption coefficients of the other, "foreign" elements. Fig. 1 is an illustration of this. In this schematic figure, curve 1 indicates the absorption of the nitrogen (X) within the wavelength range for the K-edge, curve 2 the absorption of the other "foreign" elements (q—X), and curve 3 the resultant curve of 1 + 2, i.e., the absorption curve of the whole specimen.

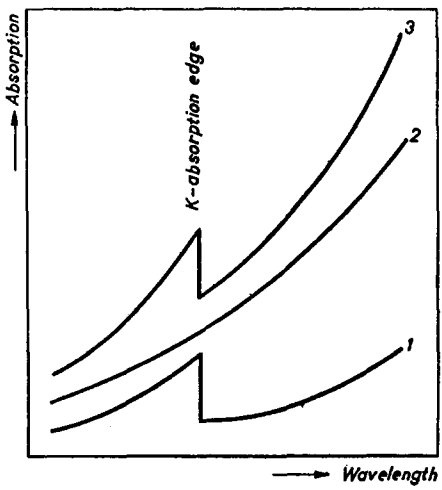

Fig. 1. The absorption of X-rays in the region of the K-absorption edge of nitrogen for a specimen of the weight q and containing nitrogen (curve 3) can be resolved into a nitrogen component X (curve 1) and a component (q–X), (curve 2) comprising the other elements. Compare the text.

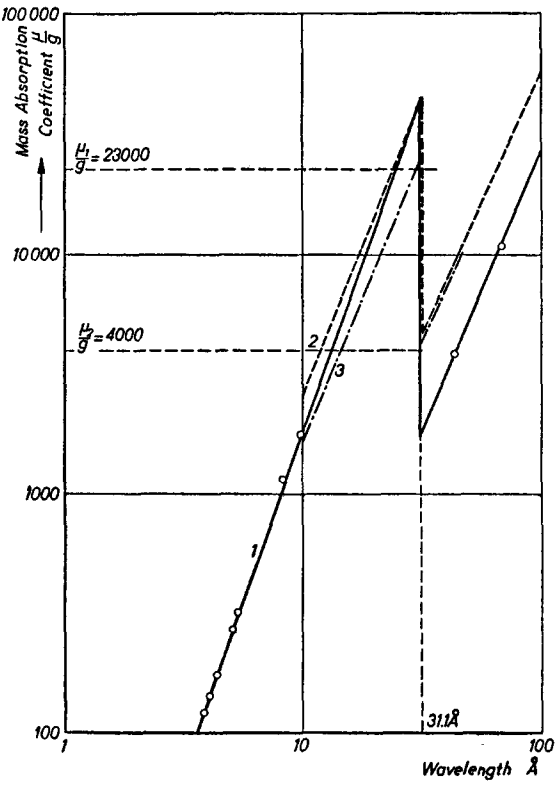

Fig. 2. The mass absorption coefficients for nitrogen in the region of the K-absorption edge. Compare the text.

Certain difficulties present themselves when it is a question of estimating the numerical values of the K-absorption jump for nitrogen, and the mass absorption coefficients on both sides of the absorption edge. In Fig. 2 are shown some absorption curves for nitrogen, which are partly based on experimental measurements and partly on calculations. The curves are presented in the double logarithmic system in order that it shall be possible to extrapolate the numerical value of the mass absorption coefficients at the absorption edge. Curve 1 is taken from experimental measurements published in COMPTON and ALLISON[4], curve 2 from measurements by HOLWECK[5], and curve 3 calculated from JÖNSSON[6] and MAGNUSSON[7]. From the curves shown in Fig. 2 it appears that it is no over-estimate if the mass absorption coefficients are taken to be 23 000 and 4 000 on the short and long wave sides of the absorption edge respectively.

References p. 433.

In the following calculations the index 1 indicates the short wave side of the absorption edge, 2 the long wave side of the edge. $\frac{\mu}{\varrho}$ indicates the mass absorption coefficient for nitrogen, and $\frac{\mu'}{\varrho'}$ the mass absorption coefficient for (q–X). $\underline{I}$ is the intensity of the incident X-rays and $\underline{i}$ the intensity of the X-rays transmitted by the specimen. X is the quantity of nitrogen in g/cm². For the two wavelengths λ_1 and λ_2 the following equations hold:

$$\begin{cases} i_1 = I_1 \cdot e^{-\left[X \cdot \frac{\mu_1}{\varrho} + (q - X)\frac{\mu_1'}{\varrho'}\right]} \\ i_2 = I_2 \cdot e^{-\left[X \cdot \frac{\mu_2}{\varrho} + (q - X)\frac{\mu_2'}{\varrho'}\right]} \end{cases} \qquad (1)$$

If the extinction $E = -\ln \frac{i}{I}$ is inserted in these equations, thus according to the above

$$E_1 = X \cdot \frac{\mu_1}{\varrho} + (q - X)\frac{\mu_1'}{\varrho'}$$

$$E_2 = X \cdot \frac{\mu_2}{\varrho} + (q - X)\frac{\mu_2'}{\varrho'}$$

the following is obtained if the analysed surface is Y cm², and λ_1 lies very near λ_2, so that $\frac{\mu_1'}{\varrho'}$ can be equated with $\frac{\mu_2'}{\varrho'}$:

$$X = \frac{E_1 - E_2}{\frac{\mu_1}{\varrho} - \frac{\mu_2}{\varrho}} \cdot Y = \frac{E_1 - E_2}{k} \cdot Y \qquad (2)$$

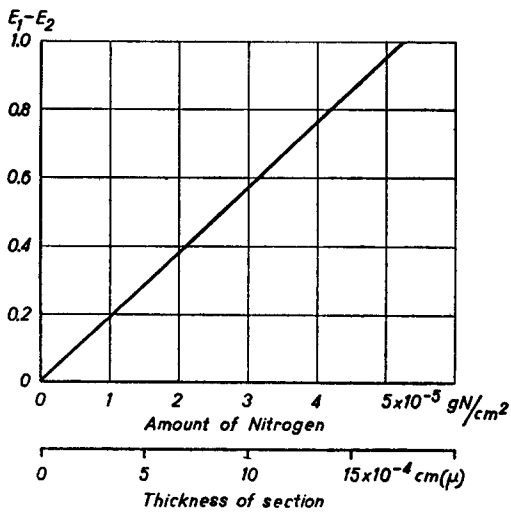

Fig. 3. The intensity difference E_1–E_2 in equation (2) as a function of the amount nitrogen in the specimen. Thickness of specimen is calculated for a tissue containing 3 % nitrogen and the spec.gravity of 1.0

References p. 433.

The quantity of nitrogen is obtained in grams. If the values of the mass absorption coefficients are inserted, i.e., k = 19000, the difference $E_1 - E_2$ can be calculated for different quantities of nitrogen. This calculation appears from Fig. 3. With the guidance of the difference $E_1 - E_2$ the smallest determinable quantity can then be calculated. If in equation 1. $I_1 = I_2$ the lower limit for what is definitely observable can be set at 5 % intensity difference between i_1 and i_2. If the specific gravity of the biological tissue is taken as 1 and the nitrogen content as 3 %, it is found, e.g., that when $E_1 - E_2 = 0.1$, it is possible to determine the nitrogen in a 1.75 μ thick section. When $E_1 - E_2 = 0.1$, if $I_1 = I_2$ there is about 10 % intensity

difference between i_1 and i_2, which is quite well measureable. In Fig. 3 both the absolute quantity of nitrogen and the section thickness of a tissue with 3 % nitrogen are drawn in.

It is of interest to establish for what quantity of nitrogen the maximum intensity difference between i_2 and i_1, i.e., the value of X, which gives maximum of the equation: $S = i_2 - i_1$. If in the equation (1) I_1 is equated with I_2 and the specimen considered to be pure nitrogen, the maximum of S is obtained (according to SANDSTRÖM[8]) for

$$X = \frac{\ln \frac{\mu_1}{\varrho} - \ln \frac{\mu_2}{\varrho}}{\frac{\mu_1}{\varrho} - \frac{\mu_2}{\varrho}} \qquad (3)$$

Cf. MAGNUSSON[7].

If the values for the mass absorption coefficients are inserted, it is found that the quantity of nitrogen which gives an maximum intensity difference is $0.92 \cdot 10^{-4}$ g/cm^2, which corresponds to a section thickness of c. 30 μ.

The calculations adduced show that there are good possibilities of determining nitrogen in thin sections of a biological material, consideration being paid to the difference between the intensities of the transmitted radiations. However, the foreign elements in the specimen (curve 2 showing (q — X) in Fig. 2) determine whether satisfactory possibilities are present for experimental determination of E, i.e. $\frac{i}{I}$. To investigate this, the mass absorption coefficients have been calculated for waterfree muscular tissue on both sides of the K-absorption edge for nitrogen. For the purpose of comparison, the corresponding values for determinations of oxygen have been included. The values appear from the following Table I:

TABLE I

THE MASS ABSORPTION COEFFICIENTS FOR MUSCLE TISSUE AT THE K-ABSORPTION EDGES FOR OXYGEN AND NITROGEN

Element	K-absorption edge	$\frac{\mu_1}{\varrho}$	$\frac{\mu_2}{\varrho}$
7 N	31.1 Å	2961	2183
8 O	23.3 Å	2421	1646

With the guidance of the values in the table, $\frac{i_1}{I_1}$ and $\frac{i_2}{I_2}$ have been calculated, and the result is shown by the curve in Fig. 4. The diagram shows that the relation between the intensity of the transmitted and the incident X-rays for nitrogen determination is most favourable, from the experimental point of view, with a section thickness between 2 and 10 μ, i.e., within a range which the above calculation also showed to be favourable. It is also possible to analyse somewhat thinner and even thicker sections in respect of nitrogen. The calculations also hold good for oxygen.

If one determines the absorption of the X-rays within an area which is $10 \cdot 10$ μ, which was shown by ENGSTRÖM[2] to be experimentally possible, one comes down to determinations of quantities of nitrogen of the order of magnitude 10^{-10} — 10^{-12} g.

The above calculations refer to monochromatic X-rays lying on both sides of and close

References p. 433.

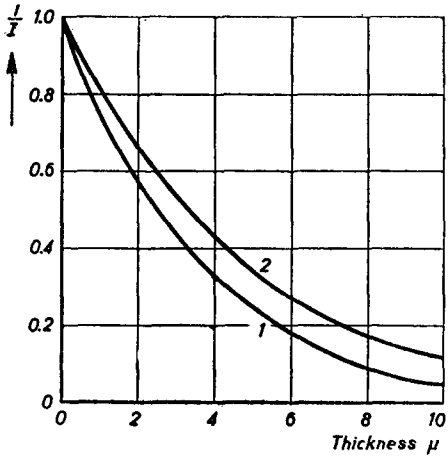

Fig. 4. The quotient between the intensity of the transmitted and incident X-rays as a function of the thickness of the tissue section. Curve 1 is calculated for the short wave side and curve 2 for the long wave side of the absorption edge. Both calculations for wavelengths close to the edge.

up to the absorption edge. Owing to the fine structure of the absorption edge and to very small shifts of the position of the edge in the spectrum due to differences in the chemical binding of the nitrogen, it is necessary to measure the absorption in wavelengths lying at a small distance from the edge. If this is taken into account equation (2) is modified as follows:

$$X = \frac{E_1 - E_2 \cdot \left(\frac{\lambda_1}{\lambda_2}\right)^p}{\frac{\mu_1}{\varrho} - \frac{\mu_2}{\varrho}\left(\frac{\lambda_1}{\lambda_2}\right)^p} \cdot Y \qquad (4)$$

λ_1 and λ_2 are the wavelengths that are used. The numerical value of the exponent p depends on what foreign elements are present. The exponent p is a function of $Z \cdot \lambda$, where Z is the atomic number. According to JÖNSSON[6] p varies continuously from 3 to 2.3 when $Z \cdot \lambda$ passes from 8 to 800. In the case of biological tissue it is of course mainly 6 C and 8 O, i.e., elements with an average atomic number of 7, which build up the foreign elements.

RESULTS OF THE CALCULATIONS

1. It is possible with X-ray absorption spectrography, making use of the K-absorption edge, to determine the nitrogen in biological material.

2. The appropriate section thickness for the analysis of a tissue containing c. 3 % of nitrogen is 2–10 μ.

3. By measuring the absorption of the X-rays on a small surface of the specimen, e.g., $10 \cdot 10$ μ, which has been shown to be experimentally possible, one can get down to analysable volumes corresponding to the size of the individual mammalian cell. Determinable quantities of nitrogen: 10^{-10} — 10^{-12} g.

4. The maximum difference in intensity when measuring on both sides of the K-absorption edge of the nitrogen is obtained at $0.9 \cdot 10^{-4}$ g N/cm^2, i.e., a section of a normal tissue about 30 μ thick.

The experimental technique and certain results will be published later.

SUMMARY

The theoretical basis for the determination of nitrogen in biological material by means of X-ray absorption spectrography is dealt with. By employing the K-absorption edge of nitrogen (wavelength 31.1 Å), it is possible to determine quantitatively the nitrogen in sections of tissue 2–10 μ thick. When an area of the order of magnitude $10 \cdot 10$ μ is used for the analysis, the analysable amounts of nitrogen are in the order of magnitude of 10^{-10}–10^{-12} g.

RÉSUMÉ

Les bases théoriques de la détermination de l'azote dans des matériaux biologiques à l'aide de

References p. 433.

la spectrographie des rayons X sont discutées dans ce travail. En tirant parti de la discontinuité d'absorption K de l'azote (longeur d'onde 31.1 Å), il est possible de déterminer quantitativement la teneur en azote de sections de tissu d'une épaisseur de 2–10 μ. Lorsqu'on utilise une surface d'environ $10 \cdot 10$ μ, l'ordre de grandeur des quantités d'azote analysables est de 10^{-10}–10^{-12} g.

ZUSAMMENFASSUNG

Die theoretische Unterlage der Stickstoffbestimmung in biologischem Material mittels Röntgenabsorptionsspektrographie wird diskutiert. Durch die Anwendung der K-Absorptionskante des Stickstoffs (Wellenlenge 31.1 Å) ist es möglich, Stickstoff quantitativ in 2–10 μ dicken Gewebeschnitten zu bestimmen. Wenn eine Oberfläche der Grössenordnung $10 \cdot 10$ μ für die Analyse angewendet wird, liegen die analysierbaren Stickstoffmengen in der Grössenordnung 10^{-10}–10^{-12} g.

REFERENCES

[2] A. ENGSTRÖM, *Nature*, 158 (1946) 664.
[3] A. ENGSTRÖM, *Acta Radiologica (Stockholm)*, Suppl. LXIII (1946).
[4] A. ENGSTRÖM AND B. LINDSTRÖM, *Experientia*, 3 (1947) 191.
[5] A. H. COMPTON AND S. K. ALLISON, *X-Rays in Theory and Experiment*, Van Nostrand N.Y. (1943).
[6] F. HOLWECK, *De la lumière aux rayons X*, Paris (1927).
E. JÖNSSON, *Absorptionsmessungen im langwelligen Röntgengebiet und Gesetze der Absorption, Diss.* Uppsala (Sweden) (1928).
[7] T. MAGNUSSON, *Investigation into absorption spectra in the extremely soft X-ray region, Diss.* Uppsala (Sweden) (1938).
[8] A. SANDSTRÖM, *Z. f. Physik*, 65 (1930) 632.

Received May 7th, 1947