A METHOD FOR THE DETERMINATION OF THE MASS OF EXTREMELY SMALL BIOLOGICAL OBJECTS

by

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I. INTRODUCTION

One of the most important problems in quantitative cytochemistry is the determination of the mass of extremely small biological objects. The ideal would be a method for weighing a single cell or a part of a cell. The masses to be determined are of the magnitude of 10^{-10}–10^{-13} g. It is necessary to be able to localize in the specimen the special cytological structure whose mass is to be determined. Thus, the weighing procedure must be performed *in situ* in a smear of cells or a microtome section of a biological tissue.

By quantitative x-ray absorption spectrography (ENGSTRÖM 1946 *etc.*) the content of a given element may be determined in grams per unit area. From this the content can be calculated in grams per unit volume of dry cell substance after measurement of the thickness of the biological sample. Since the specific gravity of individual cell structures is not exactly known, these results cannot be expressed in per cent of the total dry mass but only in grams per unit volume. Therefore, a method to determine the mass of single cell structures would be of great value. This also has the advantage of automatically correcting for the shrinkage of the specimen secondary to fixation and drying, and such shrinkage is difficult to avoid.

This paper describes a method for the determination of the mass of single cell structures by using micro-absorption measurements of x-rays.

II. PRINCIPLE

The principle of the ultramicro mass determination method can be seen from the schematic fig. 1 (*cf.* the text of the figure). By a continuous x-ray spectrum (white x-rays), passing through the Al-foil which serves as a filter, a microradiogram is taken of a biological sample, such as a microtome section or a smear preparation, and of a reference system. The reference system is a step wedge of thin absorbing foils.

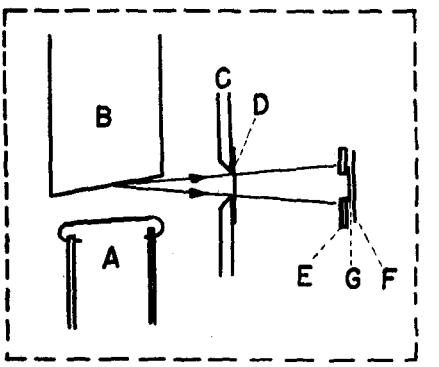

Fig. 1. Schematic representation of the method of micro determination of mass. A, hot filament cathode; B, anode; C, diaphragm; D, Al-foil (filter); E, sample holder; F, fine grained photographic film; G, sample and reference system. The whole is enclosed in high vacuum.

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The details are given in the section on apparatus and technique. In the optically enlarged image of the microradiogram the absorptions in different cell structures are compared with those of the reference system by photometric measurements. In the following section the theoretical basis of the ultramicro mass determination is presented, and proper experimental conditions, such as the voltage of the x-ray tube, suitable filter and reference system, are deduced.

III. THEORETICAL BASIS

A. The mass absorption coefficients of the biological material

To perform the following calculations it is necessary to know, for a certain wavelength region of the x-rays, the mass absorption coefficients, $\frac{\mu}{\varrho}$, of the elements constituting the biological material. (μ is the linear absorption coefficient and ϱ the specific gravity). Most of these elements have atomic numbers below 20. The mass absorption coefficients of the biological elements and other elements with similar atomic numbers are tabulated in table I for certain wavelengths. The values of $\frac{\mu}{\varrho}$ are taken from the experimental tables published in the monograph of COMPTON AND ALLISON, and the values within brackets are extrapolated.

TABLE I

THE MASS ABSORPTION COEFFICIENTS OF ELEMENTS WITH LOW ATOMIC NUMBERS FOR CERTAIN WAVELENGTHS

Element	4.36 Å	5.17 Å	6.97 Å	8.32 Å	9.87 Å	11.90 Å	13.37 Å
1 H	1.5	2.2	4.8	7.9	13	(22)	31
2 He	4.6	7.5	18	33	56	(92)	126
3 Li	19.2	32	78	130	220	(380)	530
4 Be	29.2	49	119	200	340	(575)	800
5 B	46.0	75	185	320	520	(850)	1150
6 C	97.8	160	390	656	1063	(1650)	2170
7 N	166	273	645	1109	1796	(2865)	3836
8 O	258	413	976	1589	2540	(4070)	5456
9 F	370	570	1330	2100	3000	(5030)	6950
10 Ne	478	763	1727	2750	4310	6850	8500
11 Na	600	930	2070	3300	5000	(500)	(700)
12 Mg	750	1130	2440	3900	(420)	(710)	(900)
13 Al	880	1370	2800	330	500	850	(1100)
14 Si	1100	1650	(290)	(450)	(715)	(1200)	(1600)
15 P	1330	2010	(400)	(650)	(1040)	(1700)	(2300)
16 S	1550	221	500	794	1320	2100	(2850)
17 Cl	1800	277	610	962	1570	2500	(3450)
18 A	202	324	748	1160	1860	3000	(4050)
19 K	(255)	(380)	(880)	(1320)	(2100)	(3400)	(4700)
20 Ca	(305)	(440)	(1000)	(1550)	(2360)	(3800)	(5100)
23 V	(455)	(650)	(1380)	(2110)	(3160)	(5000)	(6500)
26 Fe	610	(910)	(1750)	(2700)	(4000)	(6200)	(8300)
28 Ni	715	1150	2000	3140	4540	6900	—
29 Cu	760	1190	2130	3450	5036	7550	—

The main elements of biological materials (except calcified tissues) are hydrogen, carbon, nitrogen and oxygen. Of these hydrogen contributes least to the x-ray absorption.

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For the primary calculation the biological material is assumed to be built up of only carbon, nitrogen and oxygen. The mass absorption coefficient of various mixtures of these three elements can be calculated from the diagram in fig. 2. The diagram is valid for the wavelength 8.32 Å. The abscissa indicates the ratio between carbon and oxygen, and the ordinate the resulting mass absorption coefficient for the mixture, hereafter designated $\left(\frac{\mu}{\varrho}\right)_{CNO}$. The transverse lines indicate different concentrations of nitrogen.

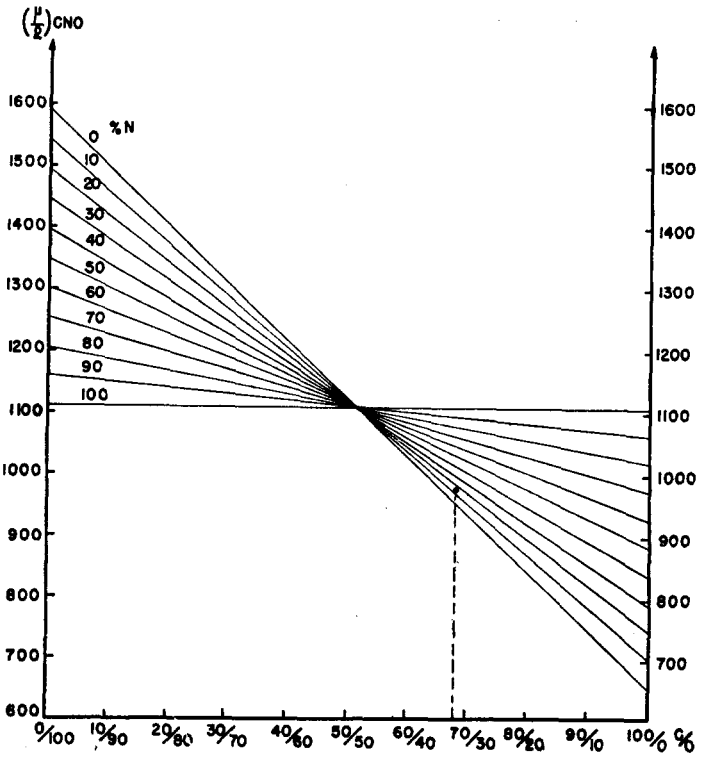

Fig. 2. The magnitude of the mass absorption coefficient, $\left(\frac{\mu}{\varrho}\right)_{CNO}$, for arbitrary mixtures of carbon, nitrogen and oxygen at the wavelength 8.32 Å. The percentage of nitrogen is calculated on the total amount of carbon, nitrogen and oxygen. The abscissa expresses the ratio between carbon and oxygen in such a way that the lines for the same nitrogen percentage become straight.

The amount of nitrogen is calculated in per cent of the total amount of C, N and O. The abscissa expresses only the ratio between C and O and not the percentages of these elements. The numerator, y, in these ratios is best expressed as:

$$y = \frac{100 \cdot \% \text{ C}}{\% \text{ C} + \% \text{ O}} \qquad (1)$$

where % C and % O are calculated in per cent of total weight. For protein of an average composition the percentages of the principal elements are: 51% C, 16% N, 24% O, 7% H and ~2% S. The mass absorption coefficient of the carbon, nitrogen and oxygen in protein, $\left(\frac{\mu}{\varrho}\right)_{CNO_p}$, is thus calculated:

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$$y_p = \frac{100 \cdot 51}{51 + 24} = 68.$$

The percentage of nitrogen must be expressed as per cent of C, N and O only:

$$\% N_{corr.} = \frac{16 \cdot 100}{16 + 24 + 51} \approx 18.$$

The $\left(\frac{\mu}{\varrho}\right)_{CNOp}$, as read from the diagram in fig. 2, is then 978.

The diagram can be used for the calculations of $\left(\frac{\mu}{\varrho}\right)_{CNO}$ also for other wavelengths. This depends on the fact that the ratios between $\frac{\mu}{\varrho}$ for carbon, nitrogen and oxygen are practically constant for different wavelengths, as is shown in table II, where $\left(\frac{\mu}{\varrho}\right)_N$ is selected as unity. Thus there is a constant proportionality between a certain $\left(\frac{\mu}{\varrho}\right)_{CNO}$ and *e.g.* $\left(\frac{\mu}{\varrho}\right)_N$, independent of the wavelength. For $\left(\frac{\mu}{\varrho}\right)_{CNO_p}$ the constant of proportionality is:

$$\left(\frac{\mu}{\varrho}\right)_{CNOp} : \left(\frac{\mu}{\varrho}\right)_N = \frac{978}{1109} = 0.88,$$

calculated at 8.32 Å, at which wavelength the mass absorption coefficients are known.

TABLE II

THE RATIOS BETWEEN THE MASS ABSORPTION COEFFICIENTS OF CARBON, NITROGEN AND OXYGEN FOR CERTAIN WAVELENGTHS

Wavelength Å	$\left(\frac{\mu}{\varrho}\right)_C : \left(\frac{\mu}{\varrho}\right)_N : \left(\frac{\mu}{\varrho}\right)_O$
4.36	0.59 : 1.00 : 1.55
5.17	0.59 : 1.00 : 1.51
6.97	0.60 : 1.00 : 1.51
8.32	0.59 : 1.00 : 1.43
9.87	0.59 : 1.00 : 1.41
11.90	0.58 : 1.00 : 1.42
13.37	0.57 : 1.00 : 1.42

Using the calculated constant of proportionality one obtains the mass absorption coefficients of "C-N-O protein" at different wavelengths. The values are shown in table III.

TABLE III

THE RESULTING MASS ABSORPTION COEFFICIENT OF THE MEAN MIXTURE OF CARBON, NITROGEN AND OXYGEN IN PROTEIN FOR CERTAIN WAVELENGTHS

Wavelength Å	4.36	5.17	6.97	8.32	9.87	11.90	13.37
$\left(\frac{\mu}{\varrho}\right)_{CNOp}$.	146	240	568	978	1580	2530	3380

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B. Calculations of the systematic errors

It can be seen from fig. 2 that a rather large variation in the ratio between carbon, nitrogen and oxygen does not affect the value of $\left(\frac{\mu}{\varrho}\right)_{CNOp}$ to any considerable extent.

However, biological materials consist of a number of elements besides carbon, nitrogen and oxygen. To what extent do these other elements affect the analytical results? We shall now calculate the influence of these elements on the result of micro mass determination by x-ray absorption measurements, using the values of $\frac{\mu}{\varrho}$ in the previous section.

The absorption of x-rays follows the law:

$$I = I_o \cdot e^{-\frac{\mu}{\varrho} \cdot m} \tag{2}$$

where I_o is the intensity of the incident x-rays and I the intensity of the transmitted x-rays and $\underline{m}$ the amount of absorbing substance in grams/cm^2. Thus

$$\ln \frac{I_o}{I} = \frac{\mu}{\varrho} \cdot m \tag{2a}$$

By absorption measurements $\ln \frac{I_o}{I}$ is obtained experimentally. For the calculations from the experimental results it is assumed that the mass absorption coefficient of the composite biological sample is the same as $\left(\frac{\mu}{\varrho}\right)_{CNOp}$. Obviously a systematic error is introduced by this assumption, as the elements other than those which have been taken into consideration above, have different mass absorption coefficients. The question is, however, to what extent these differences in $\frac{\mu}{\varrho}$ affect the final result. This effect will be shown in the following calculations.

To the "C–N–O protein" mixture, used for the calculations above, various elements will be added. Also superimposed variations in the proportions of carbon, nitrogen and oxygen to each other are considered. The absorption of CNO_p is proportional to

$$\left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot m_{CNOp},$$

where m_{CNOp} is the mass of carbon, nitrogen and oxygen in the protein. An element x, added to this mixture, has the absorption proportional to

$$\left(\frac{\mu}{\varrho}\right)_x \cdot m_x.$$

The sum of the two last formulae is the true expression for the absorption of the mixture. When calculating the mass from the experimental results, assuming that $\left(\frac{\mu}{\varrho}\right)_{CNOp}$ is the mass absorption coefficient of the mixture (CNO_p + x), an approximate value for the total mass, m_a, is obtained. We thus get two expressions for $\ln \frac{I_o}{I}$:

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$$\ln \frac{I_o}{I} = \left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot m_{CNOp} + \left(\frac{\mu}{\varrho}\right)_x \cdot m_x = \left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot m_a \qquad (3)$$

If a systematic error of $\varepsilon\%$ is allowed in the mass determination, m_a has the value:

$$m_a = \left(1 \pm \frac{\varepsilon}{100}\right) \cdot (m_{CNOp} + m_x) \qquad (4)$$

where + is used when $\left(\frac{\mu}{\varrho}\right)_x > \left(\frac{\mu}{\varrho}\right)_{CNOp}$,

and — when $\left(\frac{\mu}{\varrho}\right)_x < \left(\frac{\mu}{\varrho}\right)_{CNOp}$.

If m_a is eliminated from (3) and (4), we obtain:

$$\left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot m_{CNOp} + \left(\frac{\mu}{\varrho}\right)_x \cdot m_x = \left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot \left(1 \pm \frac{\varepsilon}{100}\right) \cdot (m_{CNOp} + m_x) \qquad (5)$$

which can be transformed to

$$\frac{m_x}{m_{CNOp}} = \frac{\pm \varepsilon}{100 \cdot \dfrac{\left(\frac{\mu}{\varrho}\right)_x}{\left(\frac{\mu}{\varrho}\right)_{CNOp}} - 100 \mp \varepsilon} \qquad (5a)$$

When $\dfrac{\left(\frac{\mu}{\varrho}\right)_x}{\left(\frac{\mu}{\varrho}\right)_{CNOp}} = 1 \pm \dfrac{\varepsilon}{100}$, we obtain $\dfrac{m_x}{m_{CNOp}} \to \infty$.

For $1 - \dfrac{\varepsilon}{100} < \dfrac{\left(\frac{\mu}{\varrho}\right)_x}{\left(\frac{\mu}{\varrho}\right)_{CNOp}} < 1 + \dfrac{\varepsilon}{100}$ the equation (5a) is not valid, because m_a between these limits cannot differ $\varepsilon\%$ from the correct value. In this interval $\dfrac{m_x}{m_{CNOp}}$ is also approaching infinity.

When ε is fixed at 5%, and the element x is varied from atomic number 1 (H) to 29 (Cu), the values of $\dfrac{m_x}{m_{CNOp}}$ in equation (5a) are graphically presented in figures 3a to 3g for seven different wavelengths. The mass absorption coefficients and wavelengths used are those given in tables I and III.

From the diagrams it can be seen that there are two maxima. One is constantly situated at the C–N–O region and has its center for a hypothetical element with the mass absorption coefficient equal to that for the C–N–O mixture, defined above. The other broadens at longer wavelengths. The increase of the breadth is most prominent on the side towards lower atomic numbers. This depends on the influence of the K-absorption edge. Elements with lower atomic numbers have their K-edges at longer

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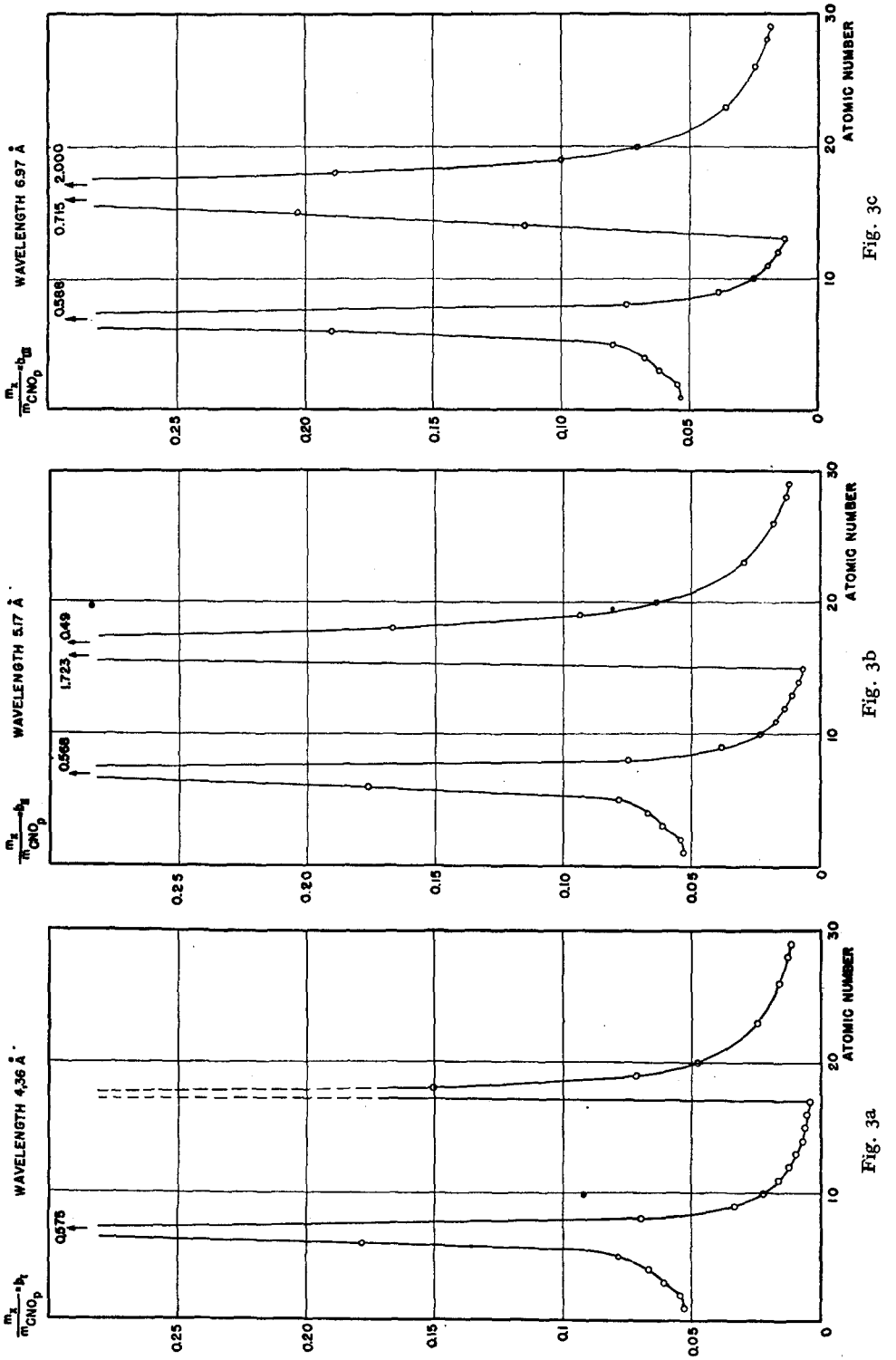

Fig. 3c

Fig. 3b

Fig. 3a

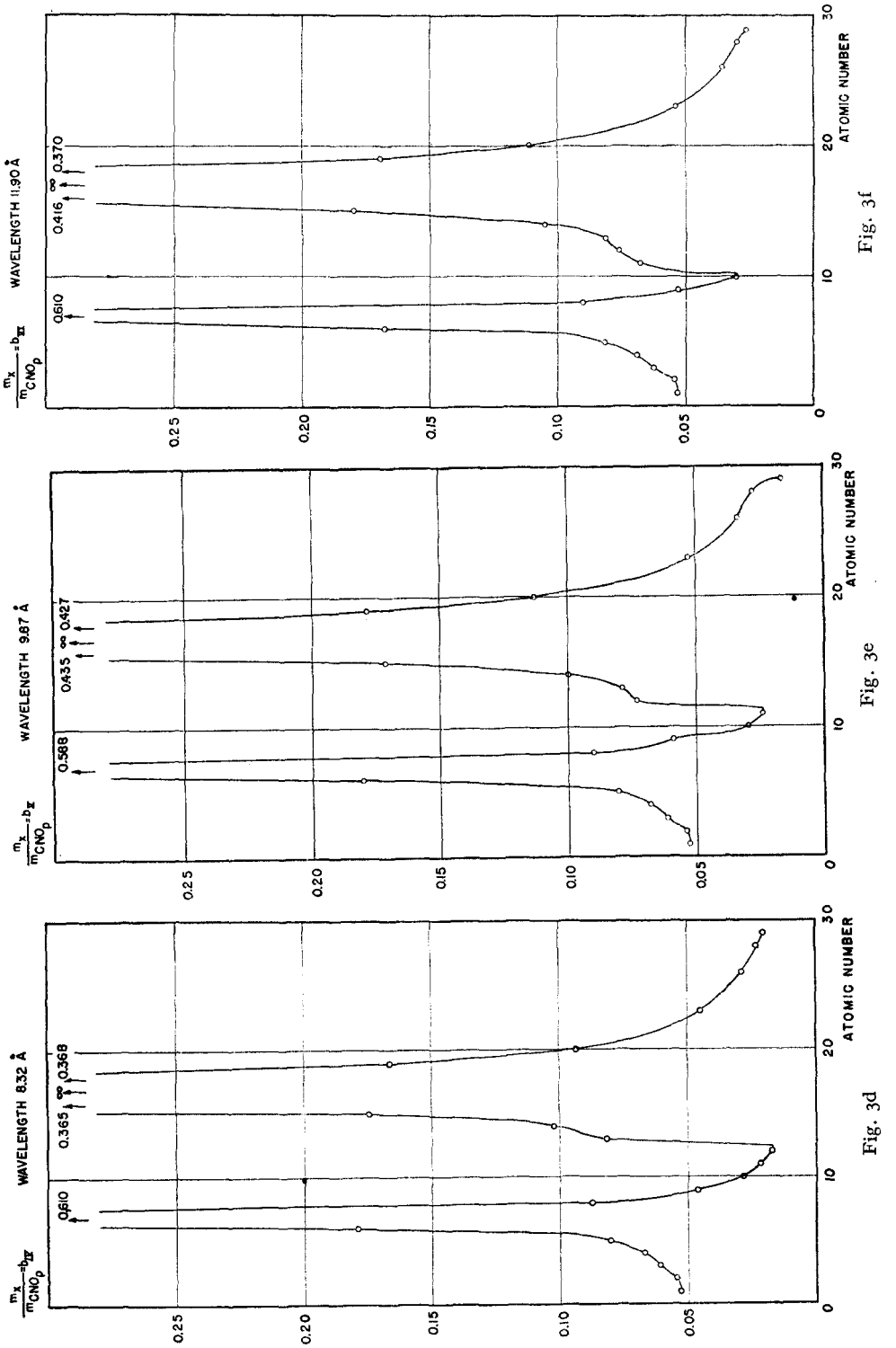

Fig. 3d

Fig. 3e

Fig. 3f

wavelengths than those with higher atomic numbers. In the parts of the curves where the derivative is positive, the systematic error ε is negative and vice versa. This fact indicates that the systematic errors for different elements can compensate each other, at least to some extent.

It is also obvious that the systematic errors will decrease if x-rays of longer wavelengths are used for the ultramicro mass determination due to the elementary composition of a mean biological sample. Therefore the position of the continuous x-rays in the spectrum must be chosen properly. The short wavelength limit of the continuous x-ray spectrum is determined by

$$\lambda = \frac{12340}{V} \qquad (6)$$

where λ is the wavelength in Ångström units and V the voltage across the tube in volts. As the intensity of the longer parts of the spectrum is small, it would be desirable to operate the x-ray tube with a relatively high voltage and filter off the short wavelength portion.

The desired filtering of the continuous x-ray spectrum, used for the ultramicro mass determination, can be performed by an Al-foil, as this element has its K-absorption edge at 7.94 Å. X-rays of wavelengths shorter than this value are strongly absorbed, and the transmission above 7.94 Å is fairly high. A suitable thickness of the filter is 9 microns, when the tube voltage is 3000 volts, corresponding to a short wavelength limit of 4.11 Å.

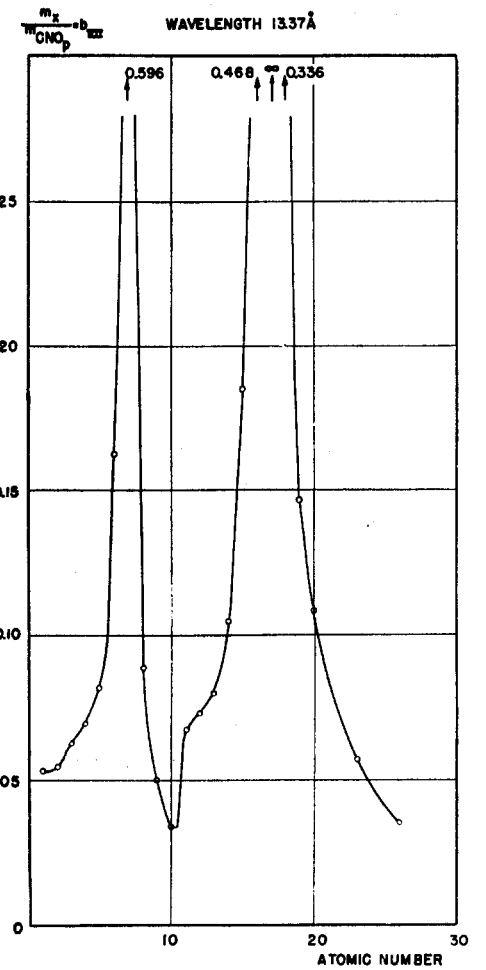

Fig. 3a–g. Diagrammatic representation of $\frac{m_{CNOp}}{m_x}$ as a function of the atomic number for seven different wavelengths. The systematic error is fixed at 5%. *Cf.* the text.

Fig. 4 shows the approximate shape of the continuous x-ray spectrum generated at 3000 volts before and after filtering through 9 μ Al. The filtering effect of the supporting collodion membrane in the preparation holder is negligible. If the relative distribution of the intensity in the filtered x-ray spectrum is estimated for certain wavelength ranges, we obtain the values in table IV. The table shows that the major part of the intensity (about 85%) lies in the region 7.65–12.64 Å, which is favourable according to fig. 3d, 3e and 3f.

A commercial x-ray tube with a beryllium window, such as MACHLETT Type AEG 50, is not suitable for the purposes discussed here, as the filtering effect is not favourable and the exposure times become extremely long for the following reasons. The LIPPMANN

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film to be used is very insensitive, and the wavelength region desired is almost completely absorbed in a beryllium window 0,5–1 mm thick.

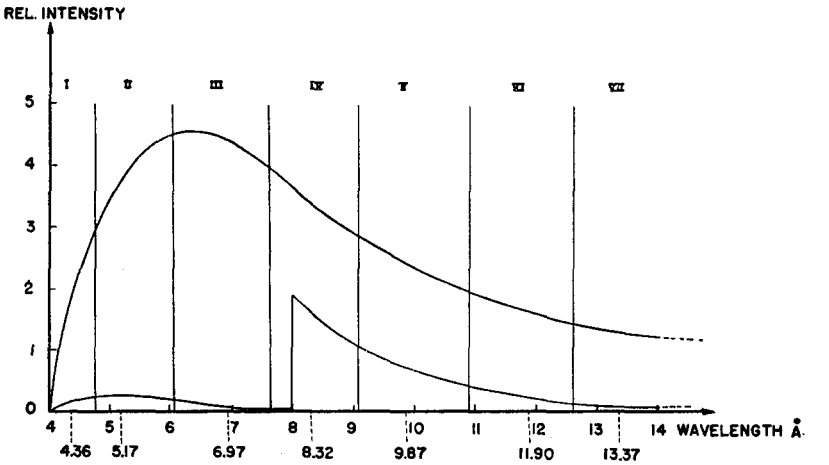

Fig. 4. The approximate shape of a continuous x-ray spectrum, generated with 3000 volts, before and after filtering through 9 μ Al. The seven wavelength regions are marked I–VII. The values of the wavelengths opposite the broken lines indicate those used for the calculations.

TABLE IV

THE RELATIVE DISTRIBUTION OF THE INTENSITIES OF THE CONTINUOUS X-RAY SPECTRUM FOR CERTAIN WAVELENGTH REGIONS. TUBE VOLTAGE: 3000 VOLTS. FILTER: 9 μ AL

Region	Wavelength Å	$a_{I\text{-}VII}$ = % of the total intensity
I	4.12–4.76	2.5
II	4.76–6.07	7.2
III	6.07–7.65	2.8
IV	7.65–9.10	38.9
V	9.10–10.89	33.5
VI	10.89–12.64	11.2
VII	12.64–∞	3.9

The next step is to calculate the limiting concentrations of the different biological elements which restrict the error to ε, taking into consideration both the intensity distribution of the continuous spectrum after filtration and the different values of the allowable percentages $\left(i.e. \frac{m_x}{m_{CNOp}}\right)$ in the different regions. Assuming a tube voltage of 3000 volts and a filter of 9 μ Al the whole wavelength region may be divided into seven parts according to fig. 4. In each partial region the percentage of the total intensity is called $\underline{a}$ (cf. table IV), and $\frac{m_x}{m_{CNOp}}$ is called $\underline{b}$ (cf. fig. 3a–3g). The division into regions is made in such a way that the wavelengths used for the calculations of $\underline{b}$ represent the approximate mean of each region. Small deviations from the mean values have no influence on the final result.

The incident x-rays with the wavelength distribution showed above passes through the sample, consisting of CNO_p plus an extra element x. Each region contributes a

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certain value of ε. The concentration of the element x that in a particular region gives $\varepsilon = 5\%$, is given by

$$\frac{b}{a} \cdot 10^4 = c \qquad (7)$$

where b and a are defined above, and c is the concentration of the element x in per cent of the mass of CNO_p. c_I–c_{VII} for the seven regions are given in the first seven columns of table V for elements of biological interest.

One region, however, has the smallest value of c, called c_{min}. This percentage (c_{min}) gives $\varepsilon = 5\%$ in that particular region. In the other regions we then obtain six other values of ε, but each of them less than 5%. These remaining values of ε are obtained from the direct proportionality which exists between c and ε according to equation (5a), where ε in the denominator can be neglected. The values of ε for c_{min} are given in the next seven columns in table V.

For c_{min} for the element x the total systematic error ($= q\%$) is the sum of the values of ε in the different regions. All wavelength regions are taken into account. As a resulting systematic error of 5% is allowed, c_{min} must be reduced with the factor $\frac{5}{q}$. *Thus* $\frac{5}{q} \cdot c_{min}$ *is the maximally allowed percentage of the element x which keeps* ε *within* 5%.

For the main elements, occurring in a biological tissue, table V shows the values which are discussed above. The values for c_{min} are underlined, and the last column gives $\frac{5}{q} \cdot c_{min}$, which is also graphically presented in fig. 5. Consideration must naturally also be paid to the different spectral sensitivity of the LIPPMANN film used. It can be demonstrated that relatively large differences in the spectral sensitivity of the film do not affect the values in fig. 5.

From table V and fig. 5 the following conclusions can be drawn. Rather large variations in the proportion of the basic elements (C, N and O) do not affect the final result to any considerable extent. The limits of variation can be wide: ~ 18% for C, ~ 60% for N, and ~ 8,5% for O. It can also be seen that the elements Na, Mg, P, S, Cl, K, Ca, and Fe may occur in concentrations which are considerably higher than their percentages in biological material in general with the exception of bone tissue, without disturbing the mass determination

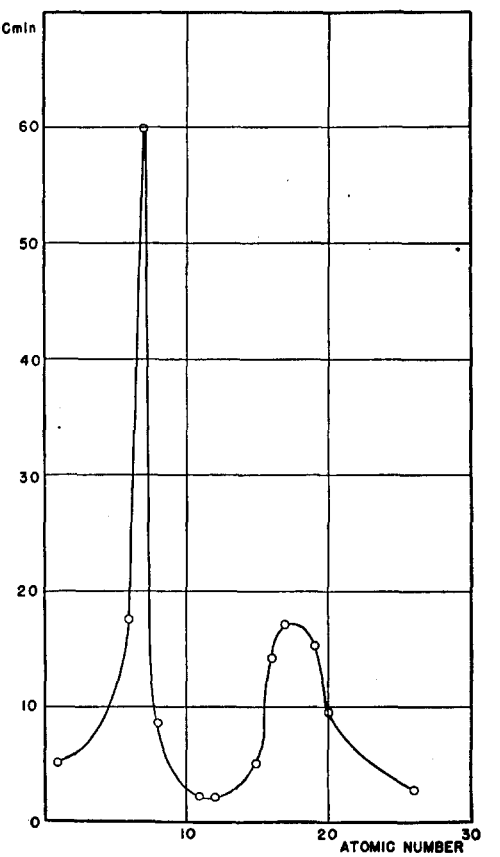

Fig. 5. The allowable percentage of the elements which gives a total systematic error of 5% in the complete spectrum (filtered x-rays). *Cf.* the text.

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by x-ray absorption measurements. Other elements with atomic numbers both higher and lower than 30 occur in biological material in such small concentrations that they do not disturb the measurements.

TABLE V

THE CALCULATION OF THE FINAL LIMIT CONCENTRATIONS OF DIFFERENT ELEMENTS OF BIOLOGICAL INTEREST FOR $\varepsilon = 5\%$. FOR EXPLANATION OF THE SYMBOLS: *cf.* THE TEXT

Element	c_I	c_{II}	c_{III}	c_{IV}	c_V	c_{VI}	c_{VII}	ε_I	ε_{II}	ε_{III}	ε_{IV}	ε_V	ε_{VI}	ε_{VII}	$\Sigma\varepsilon = q$	$c_{min} \cdot \frac{5}{q}$
1 H	213	73.7	190	13.6	15.8	47.4	136	0.32	0.92	0.36	5	4.40	1.44	0.50	12.94	5.27
6 C	715	245	676	45.8	53.7	150	417	0.32	0.94	0.34	5	4.26	1.53	0.55	12.94	17.70
7 N	2300	788	2100	157	176	545	1530	0.34	1.00	0.37	5	4.46	1.44	0.51	13.12	59.8
8 O	280	104	266	22.4	26.9	80.5	228	0.40	1.08	0.42	5	4.17	1.39	0.49	12.95	8.65
11 Na	65.3	24.6	69.0	5.52	7.07	60.8	173	0.42	1.12	0.40	5	3.91	0.45	0.16	11.46	2.40
12 Mg	49.2	19.0	55.0	4.37	21.8	67.7	187	0.44	1.15	0.40	5	1.00	0.32	0.12	8.43	2.59
15 P	24.8	9.46	725	44.9	51.0	161	475	1.90	5	0.07	1.05	0.93	0.29	0.10	9.34	5.07
16 S	20.9	2390	2560	93.7	129	372	1200	5	0.04	0.04	1.12	0.81	0.28	0.09	7.38	14.18
17 Cl	17.8	680	7150	∞	∞	∞	∞	5	0.13	0.01	0	0	0	0	5.14	17.33
19 K	288	130	357	42.8	53.3	151	376	0.74	1.64	0.60	5	4.01	1.43	0.57	13.99	15.30
20 Ca	192	88.5	252	24.0	33.7	99.2	279	0.63	1.35	0.48	5	3.56	1.21	0.43	12.66	9.48
26 Fe	64.0	25.2	93.0	7.50	10.1	31.9	91.5	0.59	1.49	0.40	5	3.71	1.16	0.41	12.76	2.94

C. The correction for hydrogen

The only element which can be expected to disturb the measurement is hydrogen (cf. fig. 5). In protein the content of hydrogen is about 8% of the mass of carbon, nitrogen and oxygen, *i.e.* m_{CNOp}. From the diagram in fig. 5 it is seen that if the percentage of hydrogen is 5.3% of m_{CNOp}, the systematic error is —5%. As there is a direct proportionality between the percentage of hydrogen and the corresponding systematic error (cf. equation (5a) where ε in the denominator can be neglected), 8% H gives a systematic error of about —7.5%. Substituting this value in equation (4) we get

$$m_a = \left(1 - \frac{7.5}{100}\right) \cdot (m_{CNOp} + m_{Hp}),$$

where m_{Hp} is the amount of hydrogen in the protein and $(m_{CNOp} + m_{Hp})$ the true total mass. The correction factor thus is

$$k_{Hp} = 1 - \frac{7.5}{100} = 0.925$$

for the hydrogen in the protein. The general expression then becomes

$$\frac{m_a}{k_x} = m_{CNOp} + m_x \qquad (8)$$

where $\frac{m_a}{k_x}$ is the corrected total mass of the sample (CNO_p + x) under investigation. The right side of equation (3) then takes the form

$$k_x \cdot \left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot \frac{m_a}{k_x},$$

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as the product must be constant, because it is equal to $\ln \frac{I_o}{I}$. Thus

$$\ln \frac{I_o}{I} = k_x \cdot \left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot \frac{m_a}{k_x} \tag{9}$$

Hydrogen produces a negative error, and the sum of the errors from the other elements will give a positive error, although not totalling 5%. The hydrogen in the calculations above refers to the protein, but there is also hydrogen in other compounds. This latter hydrogen, however, is compensated by the positive error from the other elements in the biological sample.

D. The reference system

When transferring the values of the absorption measurements to absolute values of mass the x-ray absorption of the biological material is compared with that of a standard reference system, the absolute weight of which per surface unit is known. This reference system must consist of a material with a mass absorption coefficient which has the same wavelength dependence as the CNO_p mixture. Thus foils of cellulose nitrate, consisting mainly of carbon, oxygen and nitrogen, is the most suitable material.

This principle of comparison gives two expressions for $\ln \frac{I_o}{I}$ in equation (9):

$$k_{Hp} \cdot \left(\frac{\mu}{\varrho}\right)_{CNOp} \cdot \frac{m_a}{k_{Hp}} = k_{H_{ref}} \cdot \left(\frac{\mu}{\varrho}\right)_{CNO_{ref}} \cdot m_{ref} \tag{10}$$

where $k_{H_{ref}}$ is the correction factor for the hydrogen in the reference system. m_{ref} is the true total mass of that part of the reference system which gives the same absorption as the sample being measured. Thus the mass of the biological structure is expressed in units of the reference system. The unit of this system, one foil, is determined by gravimetric methods. We get the expression

$$\frac{m_a}{k_{Hp}} = \frac{k_{H_{ref}}}{k_{Hp}} \cdot m_{ref} \cdot \frac{\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}}{\left(\frac{\mu}{\varrho}\right)_{CNOp}} \tag{10a}$$

In equation (10a) $\frac{m_a}{k_{Hp}}$ is the weight of the biological structure under investigation. m_{ref} is determined experimentally. k_{Hp} has the value 0.925 (see above) and $k_{H_{ref}}$ is determined in the same way as k_{Hp}. $\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}$ is determined from the diagram in fig. 2. $\left(\frac{\mu}{\varrho}\right)_{CNOp}$ is taken from table III. $\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}$ and $\left(\frac{\mu}{\varrho}\right)_{CNOp}$ must naturally be taken at the same wavelength, but the wavelength can be arbitrarily chosen, as there is a constant proportionality between them, independent of wavelength.

When the reference system consists of a step wedge of cellulose nitrate foils, the values of the mass absorption coefficients and of the constants $k_{H_{ref}}$ in table VI hold good for different nitrated celluloses.

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TABLE VI

CORRECTION FACTORS FOR HYDROGEN AND MASS ABSORPTION COEFFICIENTS FOR SUBSTANCES TO BE USED AS REFERENCE SYSTEM. WAVELENGTH: 8.32 A

Substance	Formula	Mol. weight	% of N	$\frac{C}{O}$	$\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}$	$k_{H_{ref}}$
Cellulose Dinitrate	$C_{12}H_{18}(ONO_2)_2O_8$	414.3	7.1	$\frac{39}{61}$	1217	0.96
Cellulose Trinitrate	$C_{12}H_{17}(ONO_2)_3O_7$	459.3	9.5	$\frac{36}{64}$	1240	0.96
Cellulose Tetranitrate	$C_{12}H_{16}(ONO_2)_4O_6$	504.3	11.5	$\frac{33}{67}$	1260	0.97
Cellulose Pentanitrate	$C_{12}H_{15}(ONO_2)_5O_5$	549.3	13.1	$\frac{31}{69}$	1274	0.97
Cellulose Hexanitrate	$C_{12}H_{14}(ONO_2)_6O_4$	594.3	14.5	$\frac{29}{71}$	1288	0.98

The nitrocellulose used for the reference system in the experiments described later had the composition: C 46.7%, N 6.6%, O 41.4%, and H 5.3%. $\left(\frac{\mu}{\varrho}\right)_{CNO_{ref}}$ is 1060 at 8.32 Å and $k_{H_{ref}}$ 0.95.

IV. APPARATUS

A schematic representation of the experimental apparatus is shown in fig. 6. The x-ray tube is evacuated by a SIEGBAHN molecular pump, backed by an ordinary two step rotating fore-pump. The anode, cathode and body of the tube are watercooled.

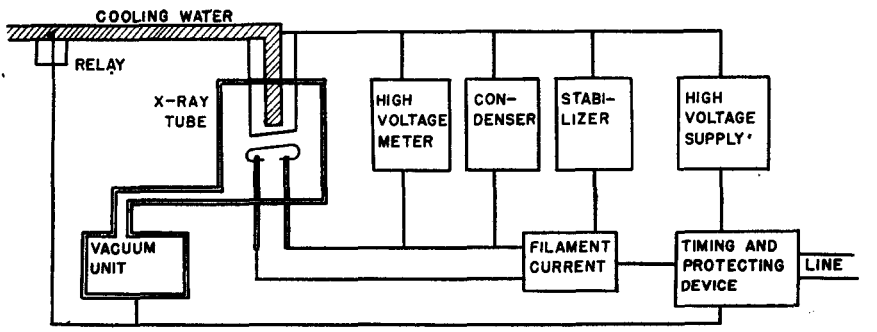

Fig. 6. Schematic representation of the experimental equipment.

The flow of water is maintained above a minimum pressure by a relay. The high voltage D.C. is obtained from the A.C. line by transforming, rectifying, stabilizing and smoothing.

A drawing of the x-ray tube is shown in fig. 7. The body of the x-ray tube, (A), is made of forged brass. The anode, (B), is isolated from (A) by means of the porcelain tube, (C). A greased cone, (D), and the metal bellow, (E), serves for adjustment of the anode with its target, (F). The target, (F), is exchangeable. Cooling water is let in at the

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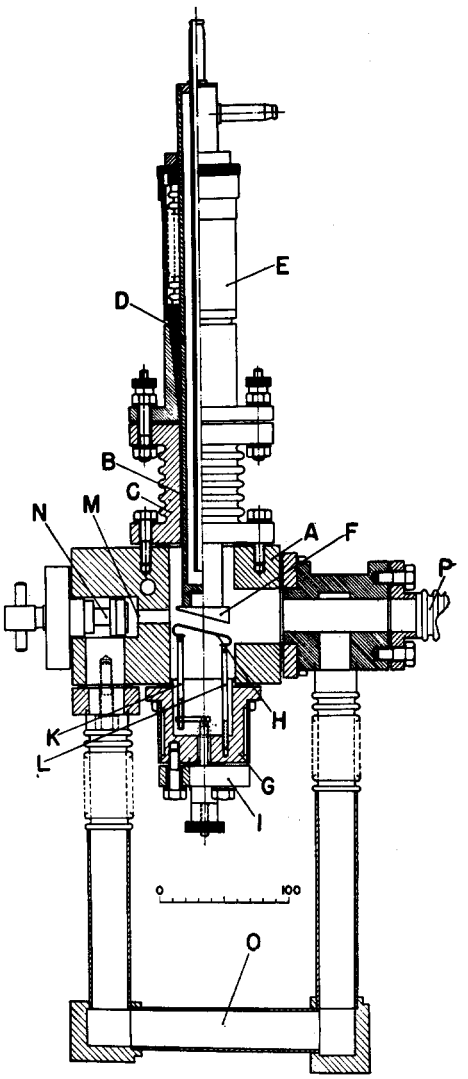

Fig. 7. The construction of the x-ray tube. *Cf.* the text.

top of the anode. The cathode, (G), is placed opposite to the anode and has a hot wire filament, (H). The filament can be adjusted with (K) and (L). (K) and (I) are isolated from the rest of the cathode. (M) is the window, consisting of 9 μ Al, and (N) is the preparation holder. The body of the tube and the cathode are grounded. All vacuum connections are made with rubber gaskets. By the vacuum connection, (O), the same pressure is maintained on both sides of the window. The vacuum connection is provided with two light traps. Evacuation takes place through (P).

A detailed drawing of the preparation holder, (N), is shown in fig. 8. The sample holder, (7), and the film are held firmly against the end of the adjustable bar, (2), by the holder, (3), which screws into (4) against the flange, (5). (6) is a tap for keeping (3) in position. (1) is the block which is fastened against the body of the x-ray tube by two screws, (8).

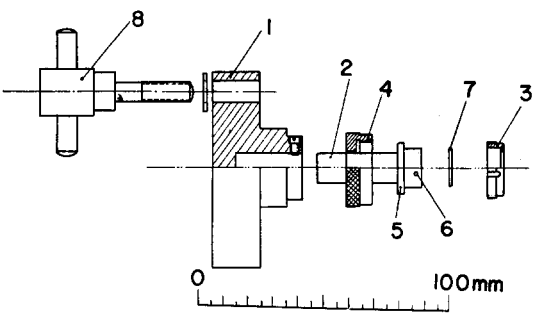

Fig. 8. The construction of the preparation holder. *Cf.* the text.

The stabilizing arrangement of the high voltage is shown in fig. 9. The high voltage equipment used had a relatively high inner impedance. Therefore the voltage will vary with the x-ray tube current. With constant filament current the emission of electrons varies, among other things, with the vacuum. In order to obtain constant experimental conditions the stabilizer changes the filament current in such a way that the high voltage is constant. The line voltage for the high voltage unit is stabilized separately. Fig. 10 shows a photograph of the equipment.

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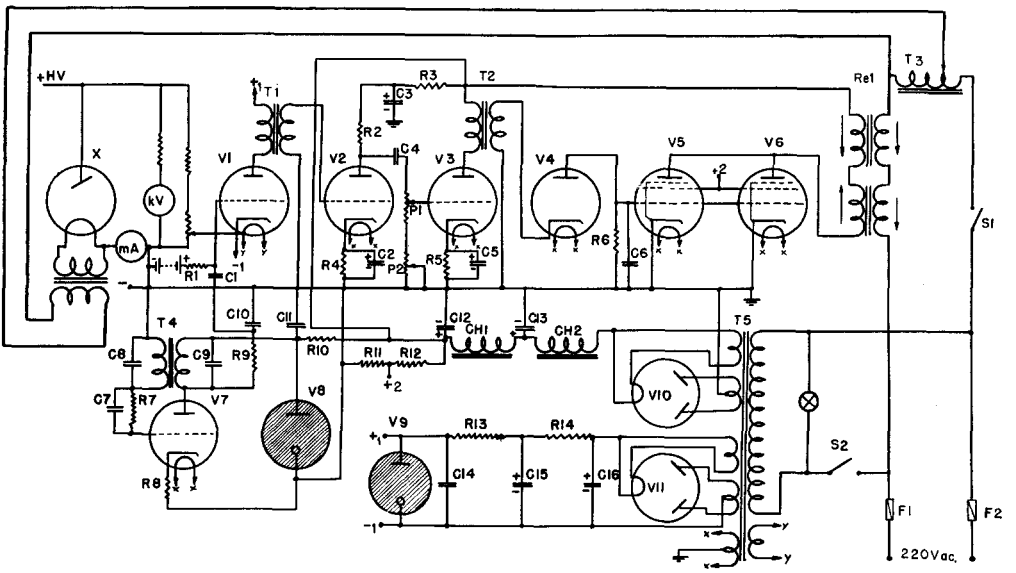

Fig. 9. Wiring diagram for the high voltage stabilizing unit.

R	Resistors	C	Condensers		V	Tubes	T	
R 1	0.5 MΩ	C 1	2000 pF		V 1	6J5	T 1	Audio frequency transformer 1:2
R 2	100 kΩ	C 2	50 μF	25 V	V 2	6J5	T 2	Audio frequency transformer 1:3
R 3	20 kΩ	C 3	30 μF	450 V	V 3	6J5	T 3	Variac transformer
R 4	1 kΩ	C 4	500 pF		V 4	6H4	T 4	Tuned transformer
R 5	1 kΩ	C 5	50 μF	25 V	V 5	6L6	T 5	Power transformer
R 6	0.5 MΩ	C 6	0.02 μF		V 6	6L6		
R 7	0.5 MΩ	C 7	2000 pF		V 7	6J5	CH 1	Choke 20 H
R 8	1 kΩ	C 8	700 pF		V 8	VR105	CH 2	Swing choke
R 9	0.5 MΩ	C 9	0.02 μF		V 9	VR105	Re 1	Reactor
R 10	7.5 kΩ	C 10	2000 pF		V 10	5U4G	S 1, S 2	Switches
R 11	10 kΩ	C 11	0.1 μF		V 11	5Y3GT	F 1, F 2	Fuses
R 12	2.5 kΩ	C 12	30 μF	450 V				
R 13	4 kΩ	C 13	8 μF	450 V	X	x-ray tube		
R 14	4 kΩ	C 14	0.1 μF					
		C 15	16 μF	450 V				
		C 16	16 μF	450 V				

V. TECHNIQUE

Careful mounting of the preparation is important. The method of mounting is briefly described with reference to fig. 11. A thin collodion foil (*ca.* 0.3–0.5 μ), laid over the slit of the sample holder, (A), serves as a supporting membrane for the preparation. The microtome section or a smear of the tissue being examined, (B), is laid on one half of that membrane. The other half of the membrane is reserved for an absorption step wedge, (C), made of cellulose nitrate foils. The foils were made from zapon varnish. The x-ray absorption of the foils should be of the same order as that of the sample. The preparation is laid against the photographic emulsion of a LIPPMANN film

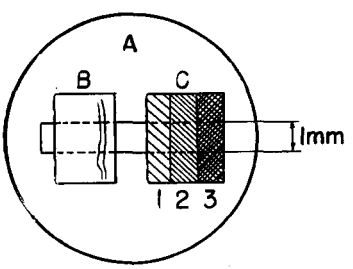

Fig. 11. The sample holder, A. B is the preparation and C the reference system.

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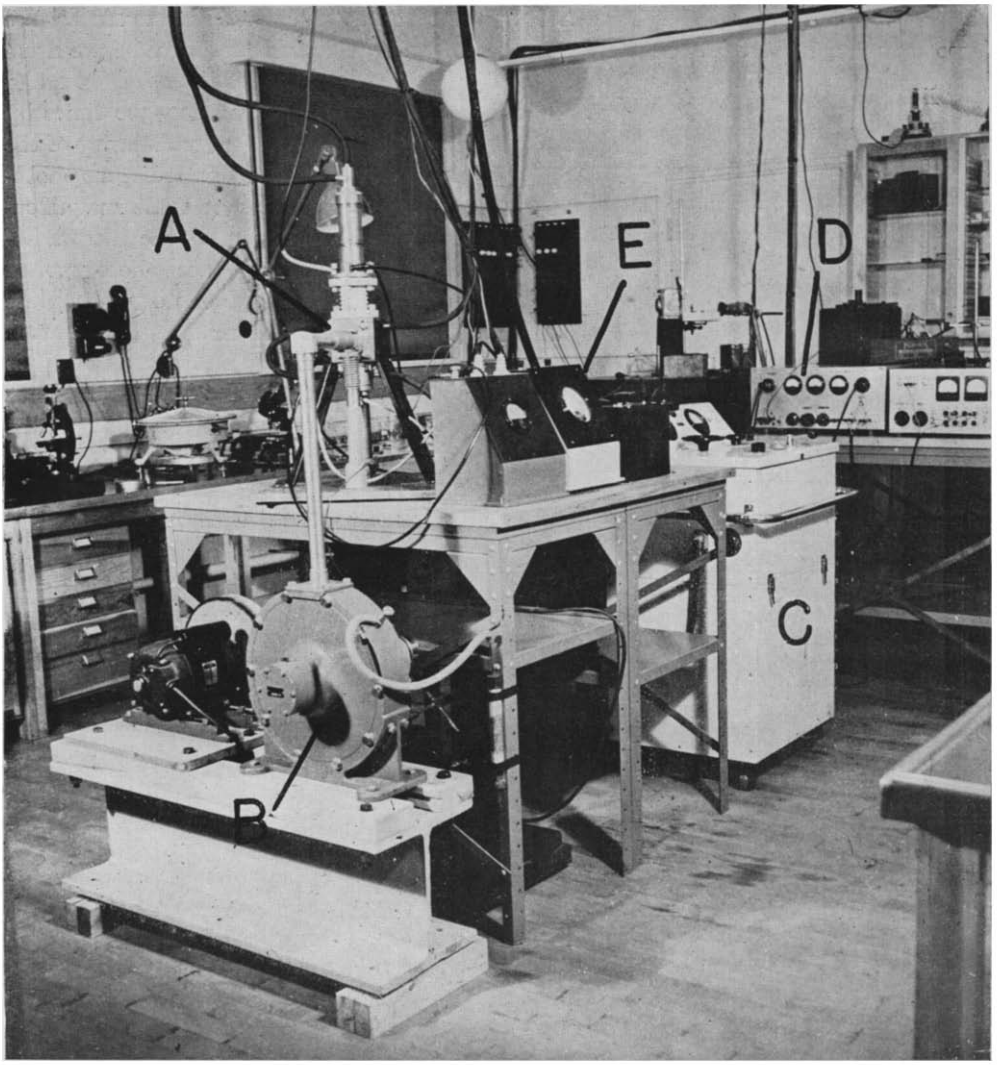

Fig. 10. View of the experimental apparatus: A, x-ray tube; B, molecular pump; C, high voltage equipment; D, stabilizing unit; E, high voltage meters.

or any other fine-grained emulsion. Very good contact must be secured. A microradiogram is then registered with long wavelength x-rays. In the following experiments the tube voltage is 3000 volts, according to the previous calculations. A check was first made, however, to control that the intensity of the incident x-rays was the same over the whole slit, so that subsequent photometric measurements could be made. The granularity of the LIPPMANN film must be kept as small as possible by proper development. The developer must give high contrast. The following developer proved satisfactory.

Metol	4 g	Sodium carbonate cryst.	110 g
Sodium sulf. cryst.	130 ,,	Potassium bromide	5 ,,
Hydroquinone	10 ,,	Water to	1000 ml

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The optimum time of development is 6 minutes at 20° C. The film is fixed in normal hypofixative and rinsed for about 30 minutes in water. The microradiograms are dried in a dustfree chamber.

The microradiogram of the sample and wedge is then enlarged 200–500 times by photomicrography. The granularity of the LIPPMANN emulsion does not disturb photometric measurements at these magnifications. The different parts of the microradiogram and the wedge are photographed separately on the same photographic plate with constant exposure time, illumination and focusing. By this arrangement it is not necessary to know either the density curve of the LIPPMANN film or that of the photographic plate when performing the subsequent photometry. Fig. 12 shows the enlarged images of a microradiogram of nerve fibres and the step wedge. In the photograph the sample is to the right and the step wedge to the left.

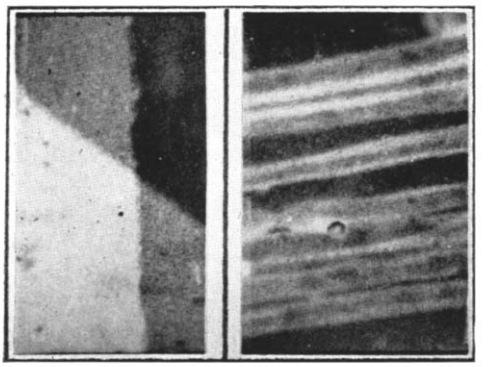

Fig. 12. A photomicrograph of a microradiogram of single nerve fibres and the corresponding step wedge. Magnification 320 ×. *Cf.* the text.

The absorptions of single cell structures are compared with those of the nitro cellulose reference system by photometric measurements on the enlarged negative. This is illustrated in fig. 13. The solid line indicates the photometer deflection as a function of the number of foils in the wedge. The dotted lines are values for cell structures. The mass of a cell structure is thus obtained in foil equivalents. The real mass of the biological sample is calculated from equation (10a). A homogeneously absorbing background of a special biological structure can easily be corrected for by subtracting the

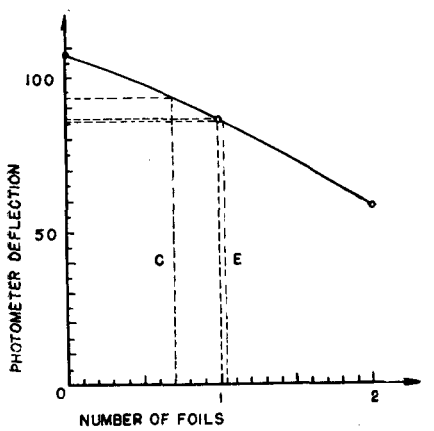

Fig. 13. The photometer deflection as a function of the number of foils in the reference system. The horizontal dotted lines indicate the photometer deflections for points in the sample. The vertical dotted lines give the foil equivalents for those points. In the figure, C indicates a measurement in the central part of a fibre in fig. 12 and E measurements in the corresponding edges of the fibre

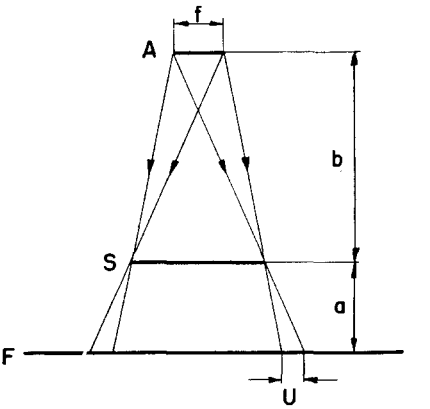

Fig. 14. Derivation of the geometrical unsharpness for a point in the sample. A, x-ray source; S, sample; F, film. *Cf.* the text.

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foil equivalent of the background from the foil equivalent of the biological structure.

The resolving power of the technique described depends on the properties of the film emulsions and the arrangement of the film, sample and x-ray source. As mentioned above the LIPPMANN emulsion has a resolving power of about 1 μ.

The geometrical unsharpness will be discussed with reference to fig. 14. If f is the size of the focal spot in the x-ray tube, b the distance between the focus and the sample and a the distance between a point in the sample and the film, we get the following expression for the geometrical unsharpness U for a point in the sample:

$$U = \frac{f \cdot a}{b} \tag{11}$$

In the following experiments the values are: f = 5 mm, b = 50 mm, and a = 0.005 mm. Then U has the value 0.5 μ, which is below the resolving power of the film.

VI. BIOLOGICAL APPLICATIONS

In the following some biological applications will be briefly discussed. A more complete result will be published later.

Fig. 15 shows a photomicrograph of a microradiogram from a 5 μ thick microtome section of human skin, from the sole of the foot, fixed in 10% formaldehyde. The paraffin was removed by xylene. From the figure it can be seen that the outer layer of the skin, *Stratum corneum*, has a stronger absorption of x-rays than have the inner layers. The limit between the epithelium and the connective tissue is also sharp. In the epithelium a higher magnification would reveal single cells and cell structures. In another section *ca.* 15 μ thick an ultra micro mass determination was performed according to the statements described earlier in this paper. The *Stratum corneum* had a mass of $7.8 \cdot 10^{-10}$ g per 100 μ^2 and the other layers of the epithelium $5.6 \cdot 10^{-10}$ g per

Fig. 15. A microradiogram of human skin from the sole of the foot. Thickness of section: 5 μ. Magnification 70 ×. 3000 volts x-rays, filtered through 9 μ Al. The white areas are more opaque to x-rays than the dark ones.

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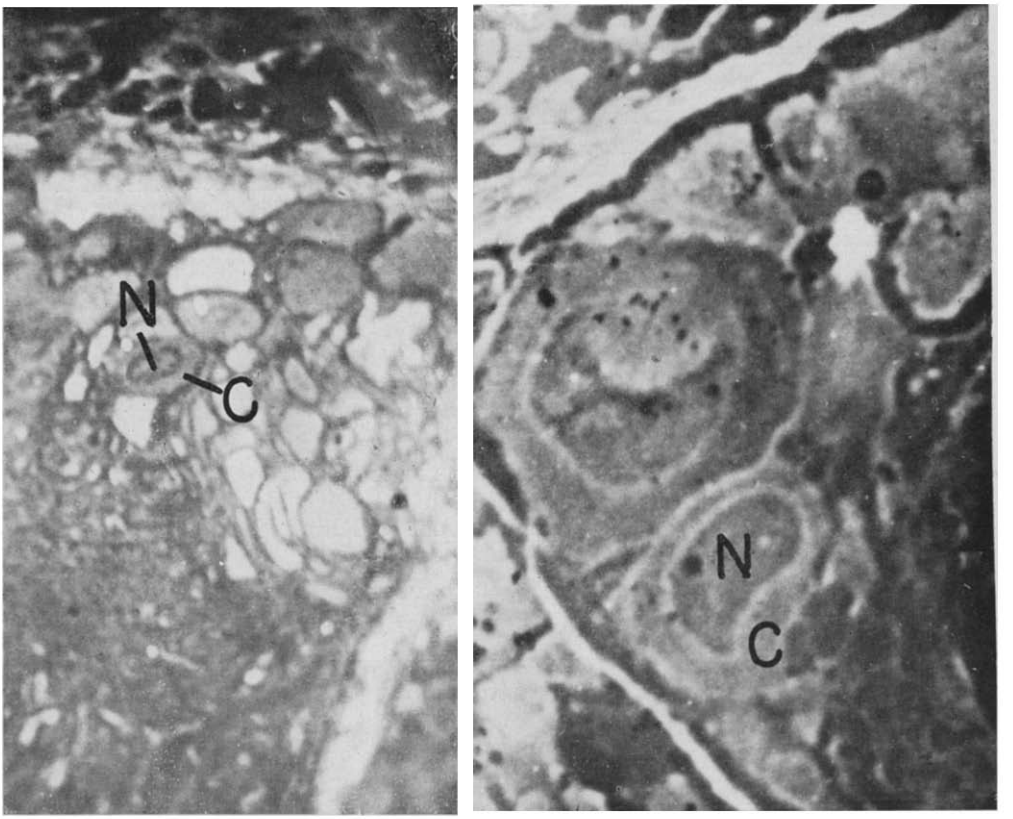

a b

Fig. 16. Nerve cells from Ganglion olfactorium in *Helix pomatia*. N, nuclei; C, cytoplasm. Thickness of section: 5 μ. Magnification: a) $\sim$ 75 ×, b) 285 ×. 3000 volts x-rays, filtered through 9 μ Al. The white areas are opaque to x-rays.

100 μ^2. The mass ratio thus was about 1.4:1 This quotient varied in skin sections taken from different parts of the body.

A material which offers the opportunity of measuring great differences in mass is nerve cells from ganglia in *Helix*. Differences occur both within the cells (nucleus-cytoplasm) and between different cells. Fig. 16 shows an enlarged microradiogram from a section of such a ganglion. The picture shows that cells vary greatly in mass and that the nucleus regularly has less mass than the cytoplasm. The quantitative data from the measurements will be published later. Cf. also fig. 17.

An example, where a great resolving power is employed, is shown in fig. 18, which is an enlarged microradiogram of single nerve fibres from the sciatic nerve in frog. These fibres have a diameter of about 10 μ. They were dissected according to the technique described by STÄMPHLI (see ENGSTRÖM AND LÜTHY 1949). From the figure it can be seen that the fibres have a greater mass in the periphery than in the center. (Cf. the text of the figure). The fibres thus have a tubular structure, where the tube wall has a greater mass than the contents of the tube. After drying quantitative measurements show that the outer layer of the fibre, which corresponds to the myelin sheath, contains

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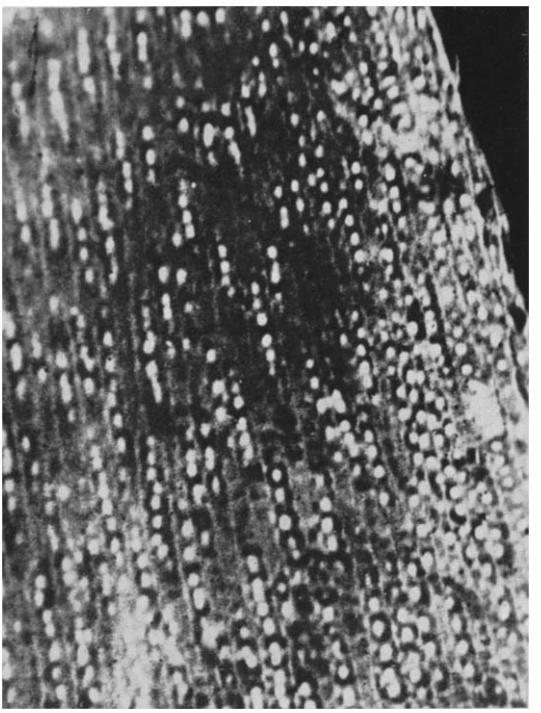

Fig. 17. A photomicrograph of a microradiogram of a 5 μ section through a growing root tip from *Allium cepa*. Magnification 110 ×. 3000 volts x-rays. Filter: 9 μ Al. The white nuclei have a strong x-ray absorption, indicating a greater mass per unit volume than the surrounding cytoplasm. Note the cell walls. In the peripheral cells the cytoplasm has a greater mass per unit volume than in the central cells.

5 to 8 times more material than the inner part, which corresponds to the axon. The outer layer has a mass of $0.3–0.4 \cdot 10^{-12}$ g/μ^3. In the picture some constrictions of RANVIER are seen.

By determining the mass in these fibres before and after extraction of lipids the amount of these could be determined. It was found that about 50% of the outer layer could be extracted. For details concerning the analyses of nerve fibres the reader is referred to the paper by ENGSTRÖM AND LÜTHY (1949).

By the method described in this paper several problems have been studied. ENGSTRÖM AND GLICK (1949) investigated the different cells in the gastric mucosa. Mass determinations in *i.a.* striated muscle fibres and chromosomes are being performed. For a survey of the x-ray techniques in general see ENGSTRÖM 1946–1949.

VII. POSSIBILITIES OF THE METHOD

It can be expected that the method of mass determination of biological material by x-ray absorption measurements will be a useful tool when solving several problems in cytochemistry. The method is being used in combination with the x-ray methods for quantitative estimation of single elements to arrive at analyses of elements referred to dry weight basis. Also, planimetric methods may be used to obtain total mass of cell parts. Absolute dry weight can be estimated where section thickness is known with sufficient accuracy.

By first determining the mass of a cellular structure in a sample and then extracting with specific solvents or treating with enzymes a repeat determination of the mass gives information about the mass of material removed.

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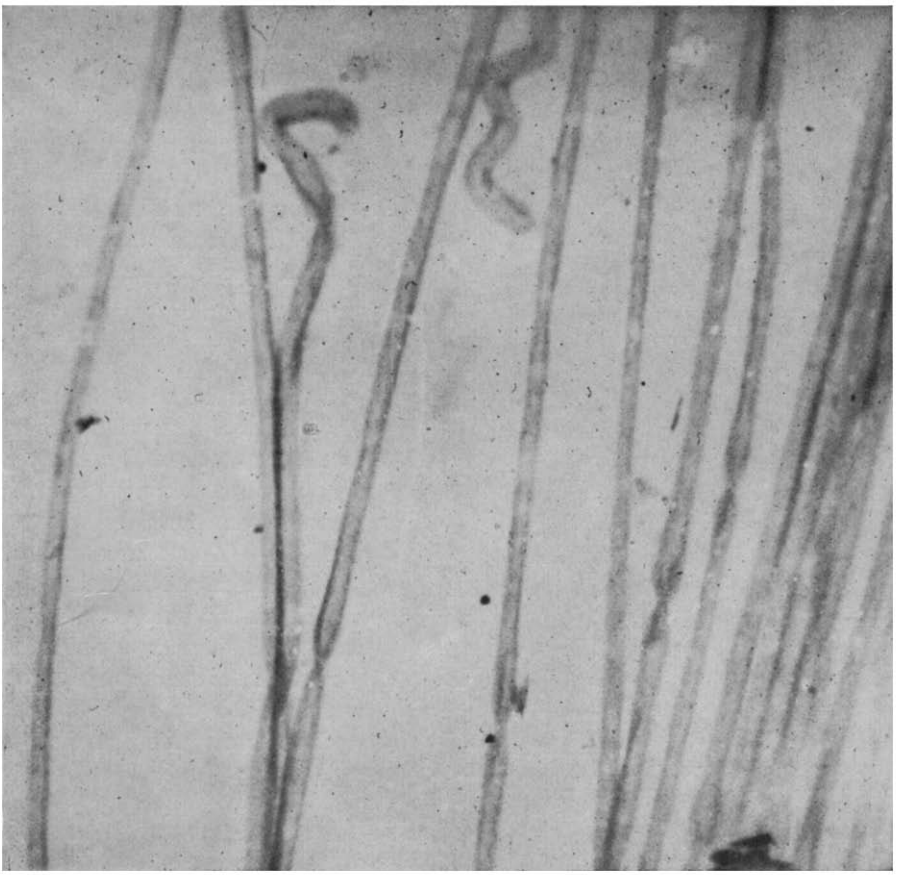

Fig. 18. Single nerve fibres from the sciatic nerve in frog. Magnification 200 ×. 4000 volts x-rays, filtered through 9 μ Al. The *black* areas are those more opaque to x-rays.

SUMMARY

A method for the determination of the mass of as small biological objects as single cell structures is described. The method is based upon absorption measurements of soft filtered continuous x-rays. From absorption data the mass can be calculated. The complete theory of the method is given. An x-ray equipment for the mass determination has been constructed and is described in detail. A number of biological applications are presented.

RÉSUMÉ

On décrit une méthode pour mesurer la masse d'objects biologiques très petits, tels que les structures d'une cellule. La base de cette méthode consiste dans la mesure d'absorption des rayons-X mous continus. A partir de ces données d'absorption on peut calculer la masse. On expose ensuite la théorie complète de cette méthode. Un appareil à rayons-X, dont on donne une description détaillée, a été construit pour ces mesures. On présente enfin quelques applications biologiques de cette méthode.

ZUSAMMENFASSUNG

Es wird eine Methode beschrieben, welche gestattet die Masse von Zellstrukturen zu bestimmen.

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Aus der Absorption weicher, filtrierter, kontinuierlicher Röntgenstrahlen lässt sich die Masse dieser Strukturen berechnen. Die vollständige Theorie der Methode wird beschrieben. Für diese Massenbestimmung wurde eine Röntgenapparatur gebaut; sie ist hier in ihren Einzelheiten dargestellt. Erste Ergebnisse an verschiedenen biologischen Objekten werden mitgeteilt.

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