ON THE ELECTRON-SCATTERING POWER OF PROTEIN STRUCTURES IN THE SPINACH CHLOROPLAST

by

M. BUSTRAAN, J. C. GOEDHEER,

Physical Institute of the State University, Utrecht

AND

J. B. THOMAS*

Biophysical Research Group, Utrecht/Delft (Netherlands)

(under the Direction of A. J. KLUYVER, Delft, and of J. M. W. MILATZ, Utrecht)

INTRODUCTION

In a preceding paper THOMAS, BUSTRAAN, AND PARIS[1] reported on the occurrence of cytoplasmic fibrils in the stroma of spinach chloroplasts. These fibrils, earlier observed by LEHMANN AND BISS[2] in the *Tubifex* egg, became clearly visible under the electron microscope after removal of the lipoid components of the stroma. Because these structures were shown to be independent of the way in which the lipoids were removed, and, moreover, since LEHMANN AND BISS obtained them in their object without any special pretreatment, the first-mentioned authors considered it rather probable that also in the preparations of the said chloroplasts the protein fibrils in question were no artefacts.

As to their shape these structures resembled chains of tiny globules—"Chromidia"—linked together by "threads"—"Interchromidia"—in a way as genes are situated in a chromosome.

Such-like globules were already demonstrated in the cytoplasm. For a survey of these studies we refer to MONNÉ[3], and to RONDONI[4]. The "Chromidia" are supposed to contain phospholipids, ribonucleic acid, and much calcium. Furthermore, they are considered to be autoreproductive and to act as "centers" for respiration and growth. In the first-mentioned paper[1] it has been remarked that, if, *e.g.*, the "globules" indeed contain heavier elements than the "threads" do, the electron-scattering power per unit of volume of the former must be higher than that of the latter.

We have set it our task to subject this aspect of the hypothesis regarding the fibril structure to an experimental test.

In our earlier investigation the ratio (a): length of "globule" shadow/length "thread" shadow in a shadow-cast preparation was determined. Next, in micrograph sof non-shadowed preparations the ratio (b): light transmission of the "globule" micrographs/

* This investigation has been made possible by a grant from the Netherlands Organisation for Pure Research (Z.W.O.).

References p. 505.

light transmission of the "thread" micrographs was established with the aid of a microphotometer. If the constitution of the "chromidia" were the same as that of the "interchromidia", the ratio (b)/(a) must be 1. As a matter of fact higher values were found. This indeed suggested a stronger electron scattering of the "globules" than that of the "threads". However, it has been emphasized that these experiments needed to be repeated in such a way that the light transmission as well as the length of the shadow were determined at the same structure. The results of this study are presented below.

Our thanks are due to Mr P. J. LANSDORP for carrying out some experiments.

THEORY

MARTON AND SCHIFF[5] presented a theory regarding the relation between thickness as well as constitution on the one side, and electron scattering on the other. For a more elaborate treatment we may refer to ZWORYKIN *et al.*[6]. In a system, represented by Fig. 1, the following formula holds for single scattering in monoatomic substances:

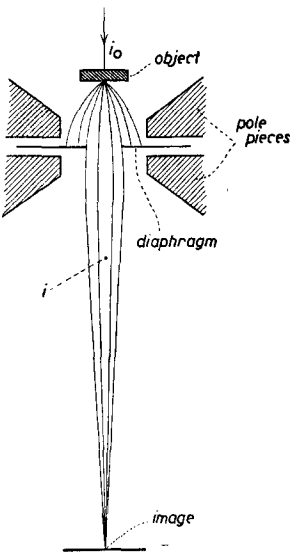


Fig. 1. Simplified scheme of the electron beam in the electron microscope.

$$i = i_o e^{-N\sigma d} \tag{1}$$

in which:

i is the transmitted current density, i_o the incident current density, N represents the number of atoms per ml, so $N = \frac{N_o \varrho}{M}$, whilst N_o is AVOGADRO's number, ϱ the density, and M is the atomic weight, σ represents the effective cross-section of one atom of the object substance for scattering outside the aperture angle δ of the objective, in our case determined by the objective diaphragm.

The above holds for monoatomic substances. If, however, more than one type of atom is present the σ's are considered to be additive functions, so:

$$N\sigma = \sum_j N_j \sigma_j \tag{2}$$

where j indicates the type of atom present in the object.

The thickness, d, of the object can be expressed as follows from equation (1):

$$d = \frac{^{10}\log \frac{i_o}{i}}{N\sigma m_e}$$

in which m_e amounts to 0.4343.

MARTON AND SCHIFF[5] described some applications of their methods. In fact, they determined the thickness of collodion films, sodium laureate curd fibres, and, *via* gelatin replicas, of etched stellite structures. The results turned out to be very satisfactory.

We decided to check the validity of MARTON AND SCHIFFS' procedure at two more structures—be it that these structures are strongly different from proteins—:

1) a crystalline structure: WO_3 crystals, and 2) an amorphous silica film. As to crystals we may remark—*cf.* ZWORYKIN *et al.*[6]—that crystalline diffraction effects may

References p. 505.

affect the results. However, in our preparations we did not notice any phenomena—intensity differences at variations of focussing, irregular reflexes on, or in the neighbourhood of the crystals—indicating such-like effects. Since, moreover, these crystals are rather thin, multiple scattering may be absent. Anticipating the results, it may be remarked here that the determination on WO_3 crystals yielded good results indeed. However, we may emphasize here that, in general, crystals are troublesome in this respect.

In the experiments to be described here we determined relative thicknesses only. However, we can add that measurements on absolute thickness, done with an interferometer technique after TOLANSKY[7], proved the validity of the above formula too. These experiments will be published elsewhere.

METHODS

i and i_0 were determined by photometrical evaluation of the densities of the photographs. To this purpose it was necessary to establish a density curve of each picture. This was done by taking a series of pictures of the same object under exactly the same conditions at exposure times of 1, 2, 4, 8, and 16 seconds, time being measured with the aid of a metronome. Since we used a Philips electron microscope it was possible to take these pictures on one film strip—Ilford direct electron recording film 5B11. By developing—with DK20—and fixating the whole strip the treatment of all pictures was exactly the same. So we were able to establish a density curve that was valid for each separate picture. So the rule of reciprocity was assumed to hold here.

After washing in running tap water for one hour the films were rinsed with distilled water before drying.

We used a magnification of 10,000 times at 100 kV and 80 kV-electrons.

By measuring the light transmission of corresponding spots in a photometer, density curves were established. The slope of these graphs, γ, proved to vary between 1.3 and 1.1. Fig. 2 shows an arbitrary density curve.

The following preparations were made.

1. a WO_3 sol in water. It was mounted on the collodion film in the usual way.

2. Silica films. In order to prepare them, the tungsten filament—diameter 0.4 mm—of the shadow casting apparatus was bent to a cup-like shape. This "cup" was filled with 3–5 mg SiO_2. Next an object glass, as used in the light microscope, was coated with a collodion film and placed at a distance of 7 cm above the "cup". Then the silica was evaporated at a pressure of 10^{-5} mm Hg applying a heating current of about 20 amps during 5 minutes. Now, the silica-covered collodion film was removed from the object glass by carefully immersing it in water; the film got loosened and floated on the water. Next, parts of the film were mounted on the object bearer of the electron microscope. Finally the collodion was washed away with acetone. During the treatment the silica film tended to curl up. This was prevented as follows. A piece of the silica-covered collodion film was picked up from the water by means of the object bearer. Then it was subsequently placed on several pieces of acetone-soaked filtering paper, until the collodion was totally removed.

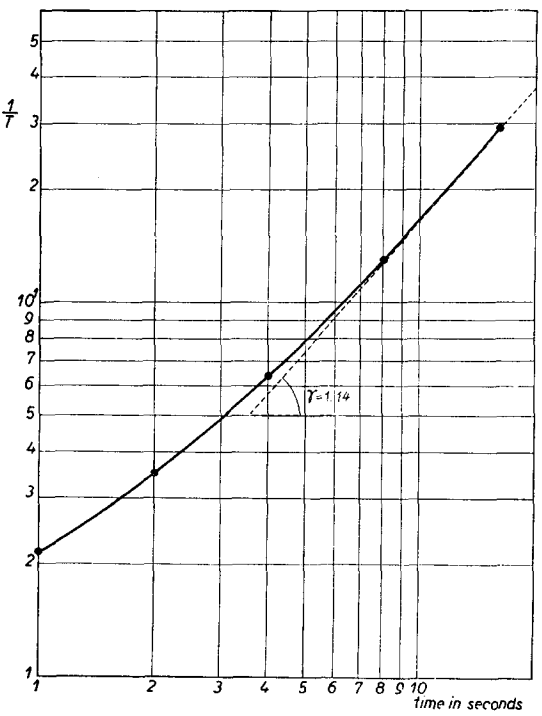


Fig. 2. Blackening curve of one of the photographic films.

3. Chloroplast preparations were made from leaves of *Spinacia oleracea*. About thirty leaves were minced in a Waring blender during one minute after addition of an equal volume of water.

References p. 505.

Next the liquid was filtered through cotton wool and centrifuged at about 1300 g subsequently. The lipoids were removed either by enzymic action or by extraction with acetone. The enzyme solution was obtained by preparing a concentrated pancreatin—O.P.G. Ed V—solution. This solution was clarified by centrifuging and—with phosphate—its pH was adjusted to 6.3. One part of the enzyme solution was mixed with two parts of the chloroplast suspension. Digestion occurred at 37° C during 5 minutes. Lipoid extraction with acetone was made by adding an equal volume of acetone to the chloroplast suspension at room temperature; the extraction lasted 10 minutes. Before mounting a drop on the collodion film, a volume of water was added twice in order to prevent the dissolving of the film.

First these preparations were photographed as set forth above. Then a survey picture was made at a magnification of 1500 ×.

Next the preparations were shadowed with a gold-manganin mixture—1:1—at an angle 1:4 and at a distance of 7 cm from the incandescent filament. In this way the electron scattering power as well as the length of the shadow could be determined at the same structure. It may be remarked that we did not observe any noticeable distortion of these structures due to the shadowing procedure.

In some cases—at long exposure times—it proved necessary to apply a correction to the photometric recordings due to a slight fog on the photographic film. Moreover, a correction was needed when comparing the light transmission of two separated spots mutually. This was necessary, because it was found that the shutter of the camera acted in such a way that during opening as well as during closing one side of the picture was exposed somewhat longer than the other one. By moving the shutter as quickly as possible the effect proved to be reproducible.

RESULTS

1. *WO_3 crystals*

Fig. 3 shows a non-shadowed WO_3 preparation. Before shadowing we determined the relative thickness d_1, and d_2 of two crystals and d_{1+2} at an overlapping spot of these crystals. We obtained:

TABLE I

RELATIVE THICKNESS OF WO_3 CRYSTALS 100 kV-ELECTRONS

c represents $N\sigma m_e$.

Exp.	$d_1 \cdot c$	$d_2 \cdot c$	$d_{1+2} \cdot c$ *calculated*	$d_{1+2} \cdot c$ *measured*	*Deviation* %
1	0.65	0.79	1.44	1.28	11
2	0.70	0.84	1.54	1.32	14

The preparation of exp. 1 was shadowed and both d_1 and d_2 were measured from the shadow length. By substituting 7.16^8 for ϱ, and calculating σ according to MARTON AND SCHIFF[5], d_1 and d_2 were computed too.

As to the computation of the value of σ we may also refer to a paper of BORRIES[9], in which a more elegant procedure has been devised. Since, however, this method is much more complicated we prefer using MARTON AND SCHIFFS' device.

The results are presented in Table II.

TABLE II

ABSOLUTE THICKNESS OF WO_3 CRYSTALS

	Thickness in A	
	measured	*computed*
d_1	280 ± 40	295 ± 30
d_2	340 ± 40	350 ± 30

References p. 505.

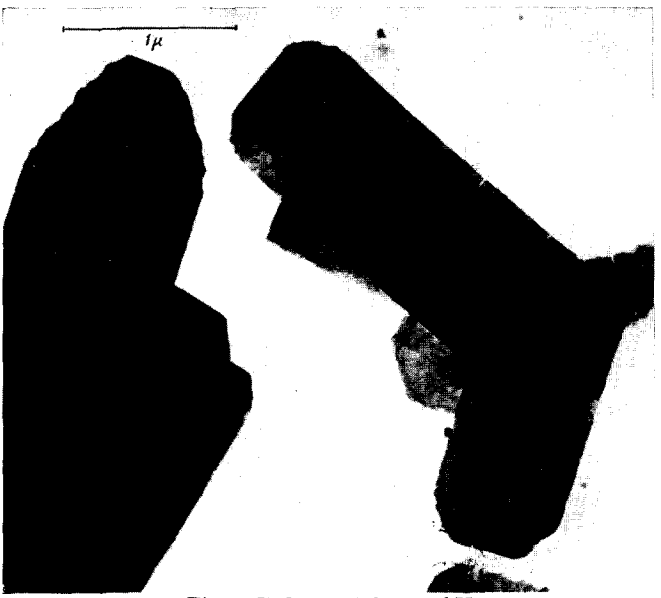


Fig. 3. WO_3 crystals. 100 kV.

2. *Silica films*

A micrograph of a non-shadowed preparation is represented in Fig. 4. The results are given in Table III.

TABLE III

RELATIVE THICKNESS OF SILICA FILMS

Exp.	*kV*	$d_1 \cdot c$	$d_2 \cdot c$	$d_{1+2} \cdot c$ *calculated*	$d_{1+2} \cdot c$ *measured*	*Deviation* %
a	40	0.69	0.75	1.44	1.39	3
b	80	0.30	0.30	0.60	0.61	2

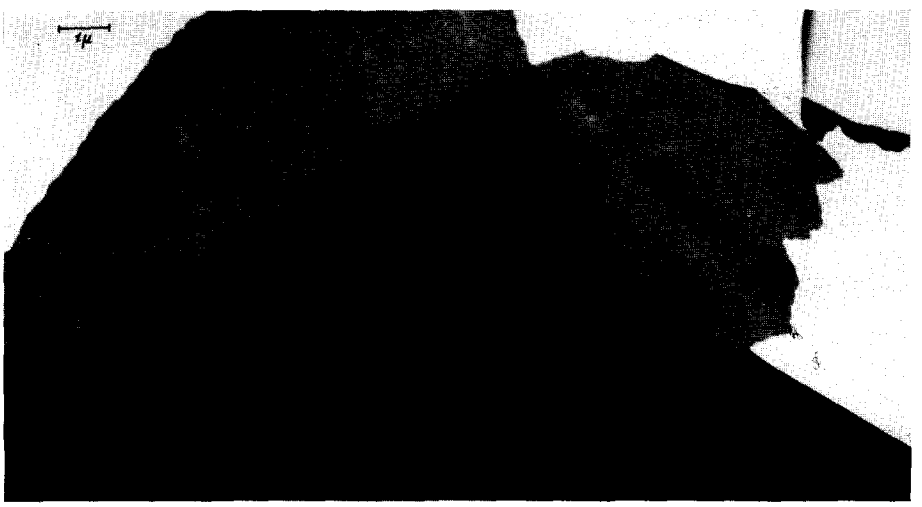


Fig. 4. Silica films. 80 kV.

References p. 505.

3. *Protein structures of Spinacia chloroplasts*

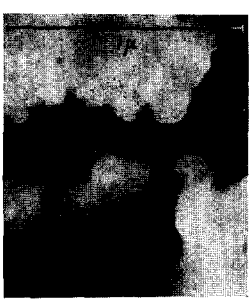
Figs. 5a and 5b show non-shadowed and shadowed preparations, respectively. The results are summarized in Table IV.

TABLE IV

RELATIVE SCATTERING CONSTANT OF CHROMIDIA—c_1—AND INTERCHROMIDIA—c_2—IN PROTEIN STRUCTURES OF THE SPINACH CHLOROPLASTS WITH SHADOW LENGTHS d_1 AND d_2, RESPECTIVELY

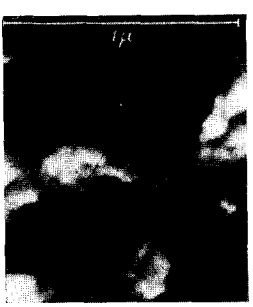
Exp.	*kV*	*Lipoids removed by*	i_1	i_2	$\frac{c_1 d_1}{c_2 d_2}$	$\frac{d_1}{d_2}$	$\frac{c_1}{c_2}$
A	80	acetone	0.137	0.310	1.63	1.25	1.31
B_1	80	acetone	0.367	0.568	1.80	1.20	1.50
B_2	80	acetone	0.440	0.620	1.72	1.26	1.36
B_3	80	acetone	0.276	0.490	1.80	1.36	1.31
C	80	acetone	0.140	0.310	1.83	1.37	1.24
D*	80	lipase	0.29	0.59	2.05	1.28	1.61
E*	80	lipase	0.41	0.66	2.12	1.33	1.59
F*	80	lipase	0.37	0.61	2.02	1.35	1.49

* These values are less accurate, since—due to a slight irregularity of the metronome—the figures, from which the density curve was derived, did not allow an exact determination of the shape of the graph. The above values refer to the most reasonable one. The extreme values for c_1/c_2 in D, E, and F respectively are: 1.23–1.82, 1.21–1.79, and 1.14–1.68.



a

Fig. 5. a: non-shadowed protein structures of the spinach chloroplast. Two "chromidia" and "interchromidia" are shown. The "horizontal" ones were measured. b: the same preparation after shadowcasting. 80 kV.



b

DISCUSSION

The determination at WO_3 crystals and at silica films showed that the sum of the thicknesses of two layers equals the thickness determined at a spot at which the layers were overlapping each other. This result proves the applicability of the technique of MARTON AND SCHIFF[5] in our objects too. It may be mentioned that, in the crystals studied, the deviation is larger than with the silica films. This phenomenon may be due to a slight multiple scattering in the crystals.

From the experiments with structures of chloroplast proteins it follows that the

References p. 505.

mean electron-scattering constant of the chromidia is 1.34 × that of the interchromidia in the preparations treated with acetone. For the "lipase experiments" this value amounts to 1.56. This clearly indicates a difference in constitution. Whether this is due to the presence of heavier elements or to a higher density in the chromidia as compared to the interchromidia cannot be decided by the applied technique.

Our thanks are due to Prof. Dr H. C. BURGER for his interest in this work and his valuable advice.

SUMMARY

The relative electron-scattering power of chromidia and interchromidia in protein structures of the spinach chloroplast was examined with the aid of the electron microscope.

It has been demonstrated that:

1. The technique of MARTON AND SCHIFF[5] holds for WO_3 crystals and silica films too.

2. The mean electron-scattering constant of the chromidia amounts to about 1.34 times that of the interchromidia in preparations treated with acetone. After lipoid removal with lipase this value is 1.56.

This indicates that the constitution of the chromidia is different from that of the interchromidia.

RÉSUMÉ

La diffraction électronique des chromides et des interchromides dans des structures protéiniques des chloroplastes d'épinards a été etudiée à l'aide du microscope électronique.

Il a été montré que:

1. La méthode d'après MARTON ET SCHIFF[5] est valable en cas de WO_3 cristaux et des pellicules de quartz.

2. La constante de diffraction électronique chez des chromides surpasse cette constante chez des interchromides par 1.34 fois en moyenne en cas d'extraction des lipides au moyen de l'acétone. Quant à l'extraction au moyen de lipase nous trouvons 1.56.

Par conséquence, la constitution des chromides a été montré differente de cette des interchromides.

ZUSAMMENFASSUNG

Mit Hilfe des Elektronenmikroskops wurden die relativen Elektronenstreuungskonstanten der Chromidien und Interchromidien des Chloroplasteneiweisses der Spinat untersucht.

Es ergab sich dass:

1. Die Methodik nach MARTON UND SCHIFF[5] an WO_3 Kristallen und Quarzhäutchen angewandt werden kann.

2. Die Elektronenstreuungskonstante der Chromidien um 1.34 mal die der Interchromidien übertrifft im Falle Extraktion der Lipoiden mittels Azeton. Nach Lipase-Einwirkung beträgt dieser Wert 1.56.

Diese Ergebnisse deuten auf eine Strukturdifferenz der Chromidien und der Interchromidien hin.

REFERENCES

[1] J. B. THOMAS, M. BUSTRAAN, AND C. H. PARIS, *Biochim. Biophys. Acta*, 8 (1952) 90.
[2] F. E. LEHMANN AND R. BISS, *Rev. Suisse Zool.*, 56 (1949) 264.
[3] F. MONNÉ, *Advances in Enzymol.*, 8 (1948) 1.
[4] P. RONDONI, *Ergebn. Enzymforsch.*, 10 (1949) 264.
[5] L. MARTON AND L. I. SCHIFF, *J. Appl. Phys.*, 12 (1941) 759.
[6] V. K. ZWORYKIN *et al.*, *Electron optics and the electron microscope*, New York 1945.
[7] S. TOLANSKY, *Multiple-beam interferometry of surfaces and films*, Oxford 1948.
[8] *Handbook of Chemistry and Physics*, Chem. Rubber Publ. Co., 13th ed. Cleveland 1946.
[9] B. VON BORRIES, *Z. Naturforsch.*, 4a (1949) 51.

Received February 19th, 1952