ON THE STRUCTURE OF THE SPINACH CHLOROPLAST

by

J. B. THOMAS*

Biophysical Research Group Utrecht–Delft, under the Direction of A. J. KLUYVER, *Delft, and of* J. M. W. MILATZ, *Utrecht (Netherlands)*

and

M. BUSTRAAN AND C. H. PARIS

Physical Institute, University, Utrecht (Netherlands)

INTRODUCTION

Many investigations on the structure of chloroplasts have already been carried out. For reviews we refer to WEIER[1], FREY-WYSSLING[2,3], RABINOWITCH[4], AND GRANICK[5]. Results obtained with the light microscope as well as studies on birefringence have provided valuable information. These data and deductions have been partly checked by experiments with the electron microscope which has made it possible to observe and to photograph submicroscopic- and even some amicroscopic structures.

Summarizing the main results emerging from a study of the literature one can state that the chloroplast:

1. is surrounded by a membrane (KNUDSON[6], ALGERA *et al.*[7], GRANICK AND PORTER[8], FREY-WYSSLING AND MÜHLETHALER[9]).

2. consists of a colourless stroma in which green grana are embedded. This holds at least for various species (MEYER[10], SCHIMPER[11], DOUTRELIGNE[12], HEITZ[13], GEITLER[14], ROBERTS[15]). The occurrence of grana has often been doubted. This was due firstly to the fact that in the light microscope chloroplasts of some species show a granular appearance only after swelling, *e.g.*, in distilled water. Secondly, in other species grana are never observed. However, due to the high resolving power of the electron microscope this question has been settled. Whereas the visibility of the grana under the light microscope is restricted to those with a diameter surpassing 0.3 μ, their occurrence has now been proved in all cases investigated.

3. may contain pyrenoids or starch grains (*cf.* MENKE[16], RHOADES AND CARVALHO[17]).

So far, however, the picture of chloroplasts of even one species is only incomplete. In the study presented here some more data are given.

MATERIAL AND METHODS

Washed leaves of *Spinacia oleracea* in 0.15 *M* phosphate buffer—pH 6.0—were grinded in a "Waring blendor" during 5 minutes. The suspension was filtered through cotton wool and centrifuged

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at an acceleration of about $1.3 \cdot 10^6$ cm/sec^2 during 5 minutes. The green upper layers of the sediment were carefully removed and transferred into a 10% saccharose solution. The suspension was washed by centrifuging and resuspending 2–3 times. Our thanks are due to Miss L. J. BARTELS for preparing several batches of chloroplasts in this way.

The preparations were mounted in the usual manner. They were studied in a Philips electron microscope. Shadow casting was performed with a gold-manganin mixture (1:1).

Extraction of the lipoids by means of benzene was carried out by mounting the chloroplasts on the preparation slide and by adding a drop of benzene, after drying the mount, renewing the drop if necessary. The duration of the extraction varied from 3–20 minutes.

Removal of the lipoids by means of lipase was effected in different ways. The enzyme preparation —consisting of a concentrated pancreatin solution—was acidified by means of diluted HCl to a p_H of about 6.0. In this manner the activity of trypsin was inhibited, whilst that of lipase was only partly reduced. In some cases the chloroplasts were fixed with 2 or 10% osmic acid solution.

The reagents were applied in different succession as follows:

1. Mounting of the chloroplasts, followed by enzyme addition; 2. fixation with osmic acid, mounting, enzyme addition; 3. mounting, fixation, enzyme addition; 4. enzyme addition, mounting; 5. enzyme addition and fixation, mounting.

If digestion had proceeded to a large extent, fixation of the material with osmic acid proved to be desirable. However, the succession of the addition of the reagents turned out to be of minor importance.

The enzymic treatment took place at 37° C during 15–40 minutes. If the chloroplasts were mounted before digestion the droplet of the enzyme solution had to be renewed several times.

Removal of the proteins was effected by using a highly purified pepsin preparation of p_H 4.0. For particulars we refer to the above mentioned description of the lipase procedure. We wish to express our gratitude towards Dr J. C. M. MIGHORST, who kindly placed the enzyme preparations at our disposal.

Ultrasonic treatment was performed in a magnetostriction oscillator. The energy delivered by the magnetostrictive tube was about 300 watts, at a frequency of 7000 Herz. Approximetely 10% of the energy was effective. The chloroplasts were treated for 1, 4, and 6 minutes. In the latter case the oscillator cup was filled with the chloroplast suspension, whilst in the experiments of shorter duration a test-tube containing the chloroplasts was attached to the vibrating cup. By the courtesy of Dr J. A. NIEMEYER these experiments could be carried out with the oscillator of the Laboratory for Physiological Chemistry at Utrecht.

RESULTS AND DISCUSSION

On the constitution of the chloroplast membrane

Fig. 1 represents a chloroplast after lipoid extraction with benzene. Since a folded bladder can be clearly seen, it must be concluded that proteins are an important constituent of the membrane. If, however, the proteins are removed by *pepsin digestion* (Fig. 2) only remnants of the membrane, composed of lipoids, are to be seen. These remnants seem to be very fragile, since they are always torn to pieces. It is tempting to draw a parallel with the erythrocyte membrane, which—according to WINKLER AND BUNGENBERG DE JONG[18], and to JUNG[19]—is composed of a lipoid outer layer—epilemma—and a protein inner layer. The proteinaceous layer is supposed to function as a bearer for the delicate lipoid film.

On the constitution of the stroma

Figs 3 and 4 show a part of a chloroplast after lipoid extraction with benzene. The proteinaceous framework can be seen between the seemingly intact grana. This is in agreement with the results of GRANICK AND PORTER. If, however, the proteins are digested by pepsin, the remaining structure is quite different (Figs 5 and 6). The sharp edges of the grana have disappeared, as demonstrated by the remnants of the grana

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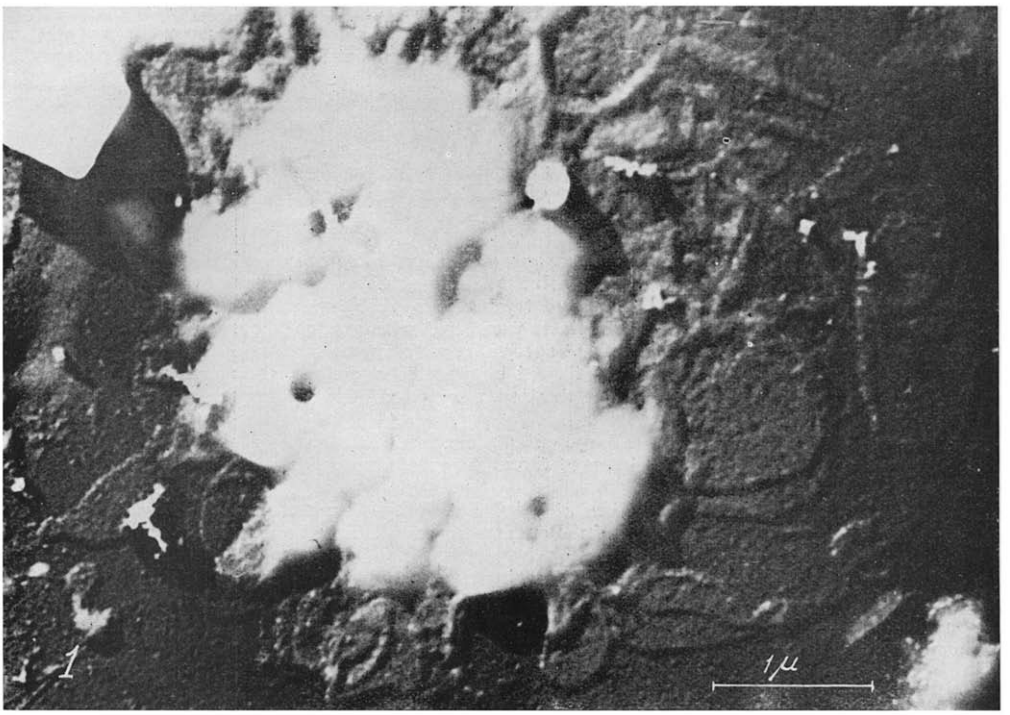


Fig. 1. Proteinaceous component of the chloroplast membrane. Benzene extraction. Shadowed. 100 kV

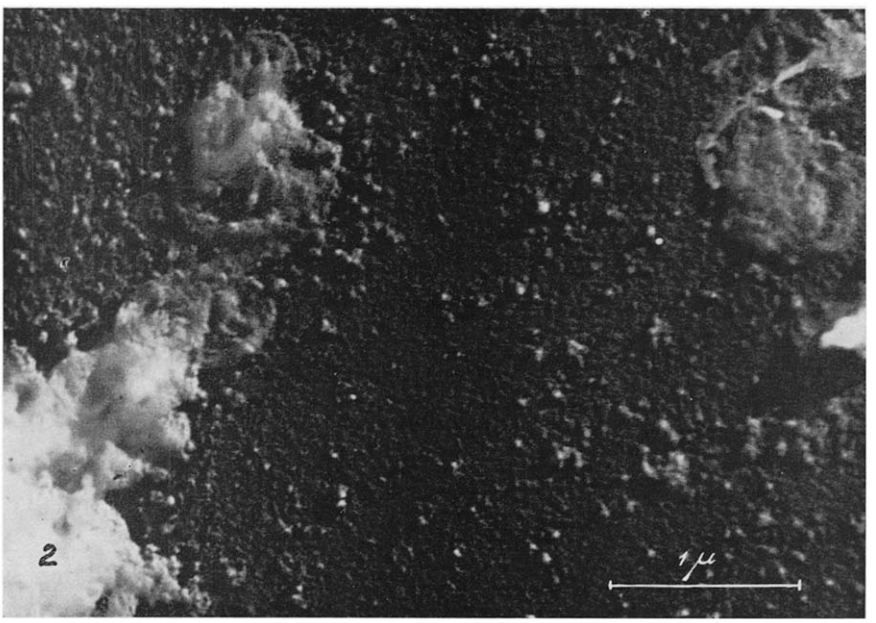
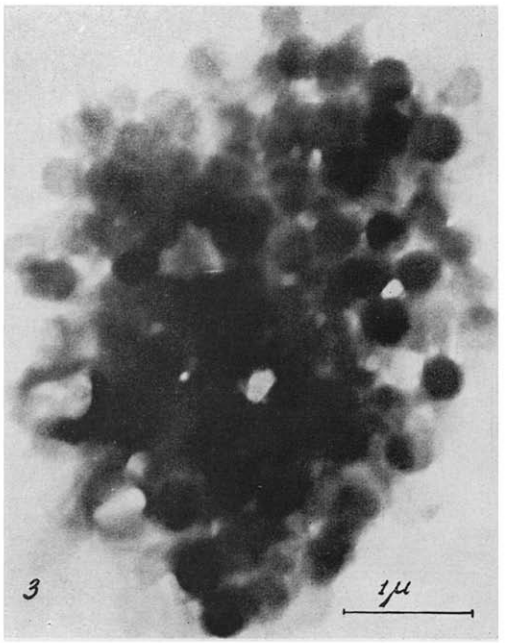
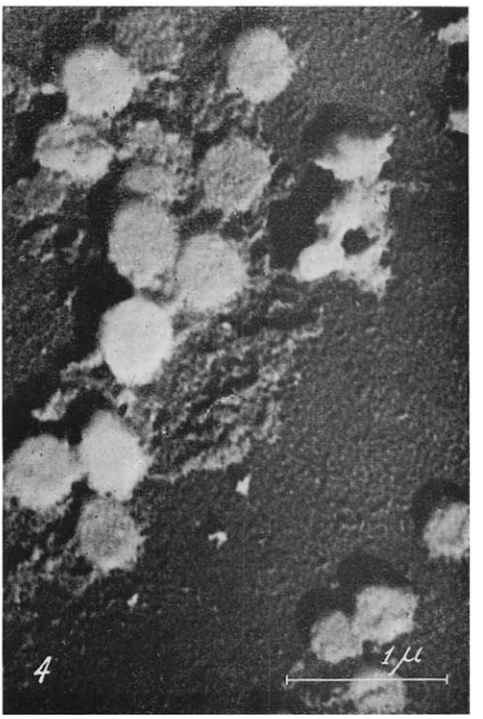


Fig. 2. Lipoid component of the chloroplast membrane. Pepsin digestion. Shadowed. 100 kV





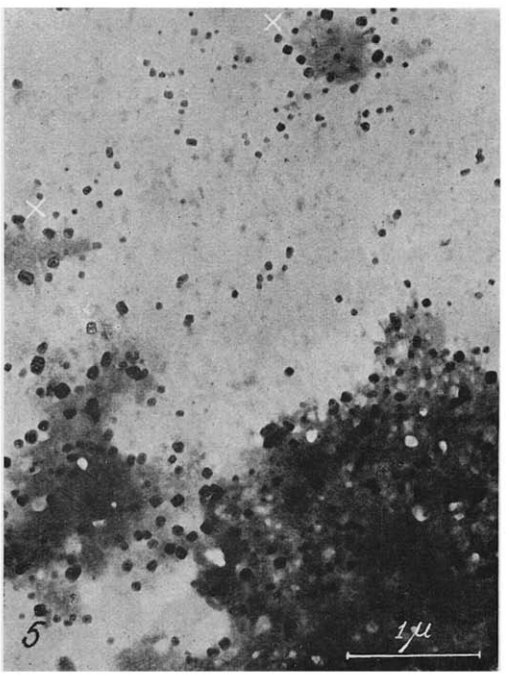


Fig. 3. Proteinaceous component of the stroma and grana. Benzene extraction. 100 kV

Fig. 4. See Fig. 3. Shadowed. 80 kV

Fig. 5. Lipoid component of the stroma and grana. Pepsin digestion. 80 kV

Fig. 6. See Fig. 5. Shadowed. 100 kV

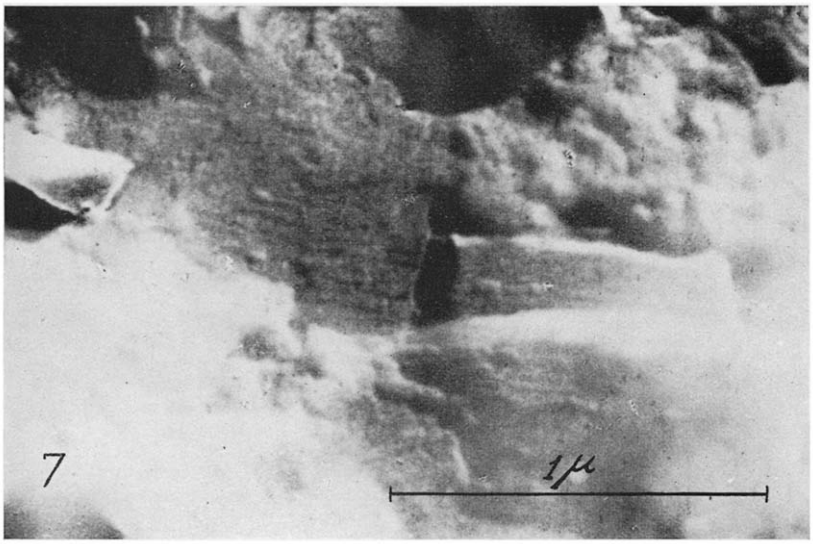


Fig. 7. Membranes with macromolecular structure in the stroma. Lipase digestion. Shadowed. 100 kV

marked X. Instead of a framework an amorphous mass of lipoids remains. In this mass open spots are always found. Possibly they represent holes remaining after the removal of the proteinaceous fibrils.

When the lipoids are digested by lipase and fixed with osmic acid, membranes of about 100 A thickness are observed. These membranes show a macromolecular design (Fig. 7). There is a striking resemblance to pictures published by FERNÁNDEZ-MORÁN[20] and representing transverse sections of the myelin sheath of the *nervus ischiadicus* of the albino rat. In our case, however, we are dealing with surface structures. The thickness of the fibrillae amounts to 100–150 A, whilst for the interfibrillar space about 50 A was measured.

After prolonged (40 minutes) lipase digestion these membranes disappeared. Probably they became too delicate to remain intact after the total removal of lipoids. A picture as represented by Figs 8 and 9 is obtained. In these experiments no osmic acid fixation was performed in order to ensure maximal lipase activity, whilst fixation of already mounted material seemed superfluous, if not harmful, for the fragile structures. However, the possibility of obtaining artefacts remains. But a comparison with electron-micrographs published by other authors (*e.g.* LEHMANN AND BISS[21]) makes us believe that here too we are dealing with naturally occurring protein structures. For reviews on this subject we refer to MONNÉ[22] and RONDONI[23].

Cytoplasmic fibrils are described as being composed of tiny globules—"Chromidia" or "Biosomen"—with a diameter of 0.1–0.2 μ, and linking threads—"Interchromidia"—, just like the genes in a chromosome. The globules contain phospholipids, ribonucleic acid and a large amount of calcium. They are said to be autoreproductive and to represent "centres" for respiration and growth. In our objects we tried to show a difference between the composition of the "globules" and the "thread" as follows. If it were true that here too the "globules" contain metals, *e.g.*, calcium, we may expect that, per unit of thickness, they cause a stronger electron scattering in the microscope than the "thread" does. In a shadow-casted preparation we determined the ratio (*a*) length of "globule" shadow/length "thread" shadow. In a preparation without shadowing we measured the light transmission of "globules" and "threads" in a photometer. From the records the ratio (*b*) absorption by

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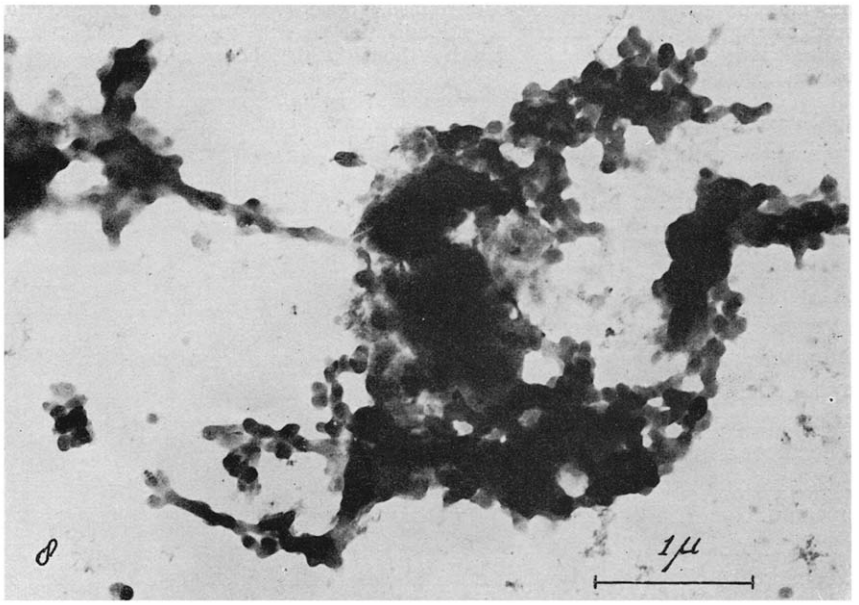

Fig. 8. Proteinaceous framework of the stroma. Lipase digestion. 80 kV

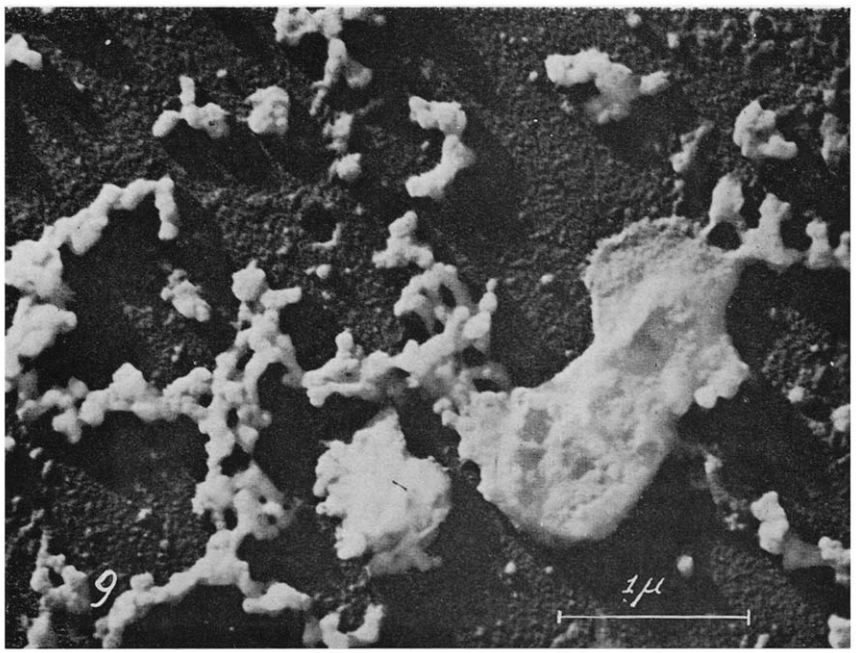

Fig. 9. See Fig. 8. Shadowed. 100 kV

"globules"/absorption by "thread" was determined. Among the values for the ratio $(b)/(\bar{a})$ we obtained figures up to 5.9. This offers a strong indication that the "globules" contain a substance which causes a larger electron scattering than the "thread" material does, and which — for instance — may be metals.

This is, of course, only a preliminary experiment. To obtain more accurate data it is necessary to study the same preparation before and after shadow-casting and to apply corrections with regard to the blackening curves of the photographic material.

Spinach chloroplasts have been chemically analyzed by several authors (*cf.* RABINOWITCH[4]). The percentages of proteins vary from 39.6 to 58, those of lipoids from 25.1 to 34, and those of the rest from 10 to 35.3. The latter values include carbohydrates. It should be mentioned here that we employed starch-free chloroplasts. We did not notice "vacuoles" in which the starch could be deposited. However, it might be possible that the pieces of membrane shown in Fig. 7 belong to such a vacuole wall. We are not yet certain on this point.

Summarizing we observed the following data concerning the stroma:

1. occurrence of a proteinaceous frame in which the grana are embedded.

2. this frame consists of "globules" linked together by a "thread" — Chromidia and Interchromidia —. The "globules" contain substances with a larger electron scattering power than those of the "thread".

3. occurrence of intrachloroplastic membranes with macromolecular surface structure.

4. after removal of the proteins a "spongy" lipoid mass remains. The holes may represent channels originally filled by the proteinaceous fibrils.

On the constitution of the grana

In agreement with data mentioned in literature (cf. GRANICK AND PORTER[8]) the spinach grana are wafer-shaped discs with a diameter of about 0.5 μ and about 0.07 μ thick. Often the parallel surfaces show a central swelling. After treatment with benzene and lipase the edges of the grana are just as distinctly visible as they are in untreated condition, *cf.* Figs 3 and 4. If, however, the proteins are removed, a quite different picture is obtained. This is demonstrated in Fig. 5 and Fig. 10. In the latter figure lipoid films which show the approximate form of the grana can be seen. The delicateness of these films is indicated by the fact that they are not visible in unshadowed preparations (Fig. 5).

From these data we conclude that the membranes of the grana are also composed of at least two components: proteins and lipoids. Furthermore we obtained the impression that these substances are arranged in two layers, the lipoids forming the outer layer. This is deduced from Fig. 11, which gives a result which is often found after lipoid extraction, in this case with benzene. Many grana are detached from the stroma, leaving round holes behind. These holes have already been described by WIELER[24], who observed them in chloroplasts extracted with alcohol and concluded that the grana are composed of fatty substances. Apparently the detached grana could not be seen under the light microscope. Of course, it does not follow from Fig. 11 that lipoids constitute the outer layer of the grana. It may be possible that the grana are embedded in lipoids belonging to the stroma. However, as mentioned above, pictures as those represented in Fig. 10 are in favour of the view that lipoids compose the outer layer of the granar membrane.

According to FREY-WYSSLING AND MÜHLETHALER[9] the grana are constructed of alternating protein- and lipoid lamellae. In one of their pictures the protein lamellae are

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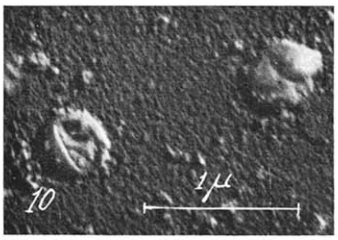

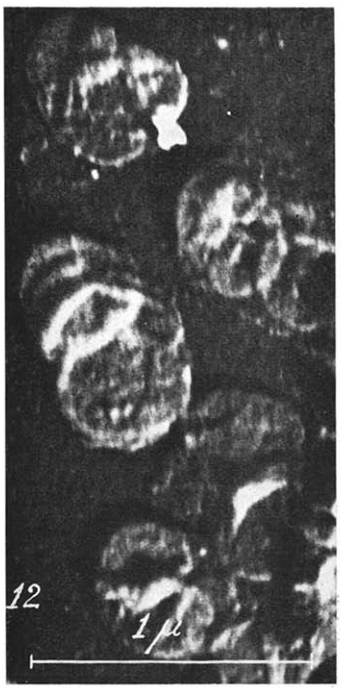

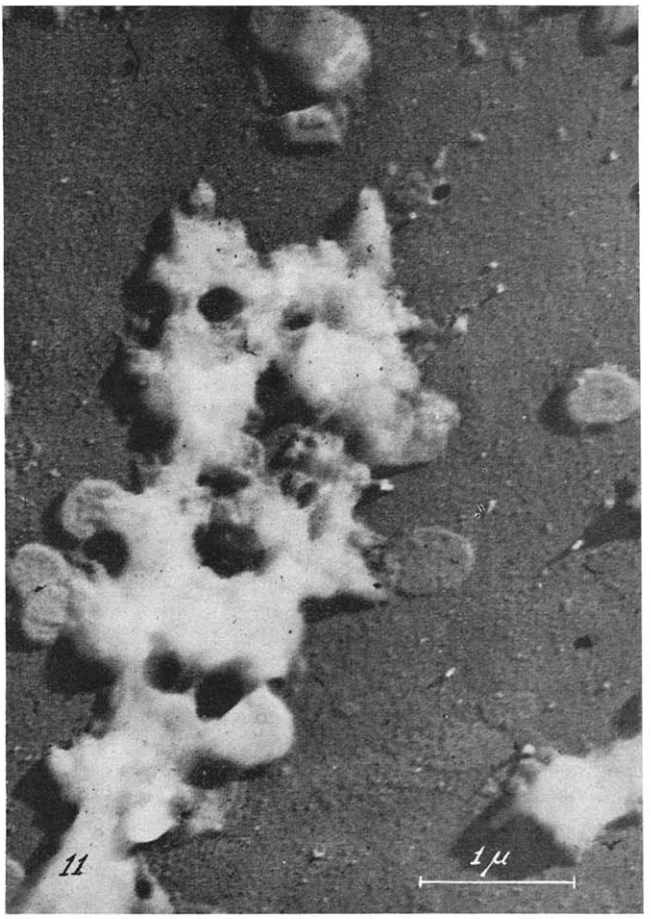

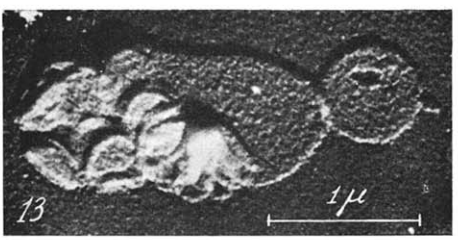

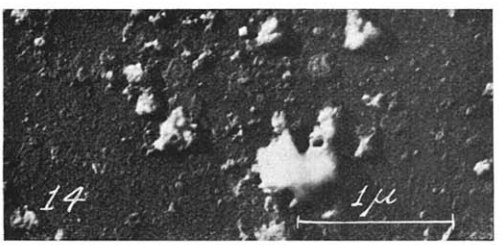

Fig. 10. Lipoid component of the granar membrane. Pepsin digestion. Shadowed. 100 kV

Fig. 11. Evidence of a lipoid outer layer of the granar membrane. Benzene extraction. Shadowed. 100 kV

Fig. 12. Granar lamellae. 4 minutes' supersonic treatment. Shadowed. 80 kV

Fig. 13. Granar lamellae and myelin formation. 4 minutes' supersonic treatment. Shadowed. 80 kV

Fig. 14. Small granar lamellae. 6 minutes' supersonic treatment. Shadowed. 80 kV

clearly shown. It was deduced from their intact form that they must be protein layers since lipoid layers should have combined to form myelin figures. The protein lamellae are shown also in the paper of GRANICK AND PORTER[8] (their Fig. 5) although they are not recognized as such.

We succeeded in dislodging these lamellae by brief exposures—1 to 4 minutes—to ultrasonic disintegration of the grana (Fig. 12). In some cases, as is shown for instance in Fig. 13, the lamellae are found in the presence of myelin figures which may be due to fusing of lipoids in the grana. Sometimes one or two lamellae are much smaller —down to about ¼ size—than the others of the same granum. It may be possible that these lamellae are responsible for the previously mentioned swelling on the granar surface. After six minutes' exposure to ultrasonic vibrations the grana seem to be thouroughly destroyed except for these small lamellae (*cf.* Fig. 14). It is difficult to state this with certainty; they may be fragments of larger ones. However, their circular shape makes us believe that we are dealing here with intact units. Maybe these minute lamellae are meant by Miss ROBERTS[25], when describing "primary granules" which would be composed of successively smaller units down to "senary granules". In our opinion, however, granar lamellae are not composed of sub-units, but a few seem to be smaller than the majority.

Cylindrical organs which are built up of transverse lamellae and surrounded by a membrane are also found in zoological material. We refer to the outer segments of the retinal rod of the guinea pig eye as described by SJÖSTRAND[26]. This author demonstrated that lipoid "knobs" are situated between pairs of discs. The diameter—2.2 μ—of the discs is much larger than that of the granar lamellae. The diameter and the height of the "knobs" are 50–250 A and 70 A respectively. We did not succeed in demonstrating the presence of such "knobs" in the grana beyond possibility of doubt. If they do indeed exist, they probably are much smaller than the retinal "knobs".

Sometimes we noticed typical artefacts in preparations treated with pepsin. This is shown in Fig. 15. In two remnants of grana fan-wise oriented lipoid rods are to be

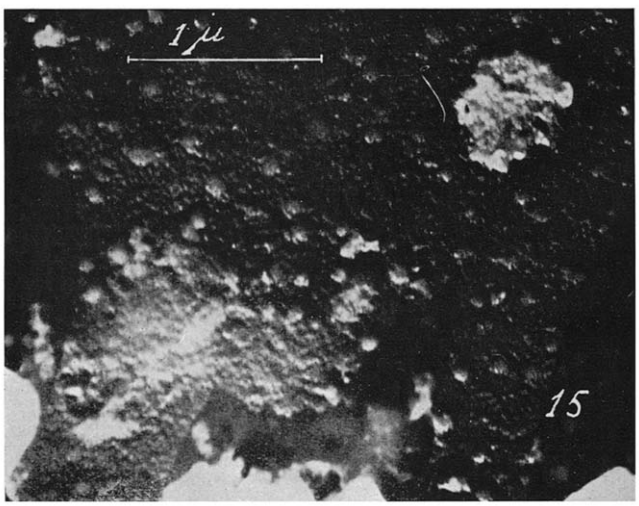

Fig. 15. Granar lipoid artefacts. Pepsin digestion. Shadowed. 100 kV

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seen. They look like regular structures, but a paper of FERNÁNDEZ-MORÁN on structures in vertebrate nerves convinced us that they are artefacts. This author could state that after trypsin digestion a structure appears which " . . . . is due to disintegration of the ordinarily compact lamellae into composite granules consisting of 50–100 A high, rod shaped single elements, which stain intensely with osmic acid. Apparently the trypsin digestion has divested the lamellae of certain (protein) components which bind the lipoid particles together".

Finally we recorded electron diffraction diagrams of a chloroplast, of a piece of isolated stroma, and of a single granum. In the stroma record no diffraction rings occurred. The chloroplast diagram showed a ring corresponding to a distance of 1.77 A. One ring was also visible in the granum diagrams. The corresponding distances are 2.01 and 2.06 A. No rings are found in diagrams of purified chlorophyll *a* and chlorophyll *b*. It should be remarked that the circumstances under which the preparations are made, may influence the result. In the first place we mention the velocity of drying. So it is difficult to interpret these data. We only can state the occurrence of distances in the order of a few A in the grana and their absence in the stroma. They might be due to amino acids.

Our thanks are due to Dr J. C. SCHOONEN for his kind help in evaluating the diagrams.

Summarizing, the conclusion can be drawn that the grana are built up of:

1. an outer membrane which is most probably composed of a lipoid outer layer and a proteinaceous inner layer.

2. protein discs—confirming the data of FREY-WYSSLING AND MÜHLETHALER[9]—with lipoids arranged in between. So far the exact arrangement of the lipoids could not be ascertained.

SUMMARY

The structure of spinach chloroplasts was investigated with the aid of the electron microscope. It has been established that:

1. the outer membrane of the chloroplasts is composed of both proteins and lipoids.
2. the stroma is also built up by these components.
3. within the stroma membranes with surface design occur.
4. a proteinaceous frame—which most probably is no artefact—occurs in the stroma. This framework strikingly resembles that found by LEHMANN AND BISS[21] in the *Tubifex* egg.
5. the grana are surrounded by a membrane composed of proteins and lipoids. Some evidence has been obtained that these substances occur as an outer lipoid layer and an inner proteinaceous layer.
6. in agreement with the results of FREY-WYSSLING AND MÜHLETHALER[9], the grana are furthermore composed of protein discs and lipoids.
7. grana showed one ring in electron diffraction diagrams corresponding with distances of about 2 A.

RÉSUMÉ

La structure des chloroplastes d'épinards a été étudiée à l'aide du microscope électronique. Nous avons montré que

1. la membrane du chloroplaste est constituée par des protéines et des lipoïdes.
2. le stroma est également constitué par ces deux substances.
3. des membranes, qui présentent une structure superficielle, sont observées dans le stroma.
4. un réseau de protéines, probablement pas un artéfact, et ressemblant étonnamment à la structure trouvée par LEHMANN ET BISS[21] dans l'oeuf de *Tubifex*, est rencontré dans le stroma.

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5. les grains sont entourés d'une membrane constituée par des protéines et des lipoïdes. Probablement les lipoïdes sont forment la couche superficielle.

6. en accord avec les résultats de FREY-WYSSLING ET MÜHLETHALER[9], les grains contiennent en outre des disques protéiniques et des lipoïdes.

7. des diagrammes de diffraction électronique des grains isolés présentent un cercle correspondant à une distance d'environ 2 A.

ZUSAMMENFASSUNG

Mit Hilfe des Elektronenmikroskops wurde die Struktur der Spinatchloroplasten untersucht. Ergebnisse:

1. Die Aussenmembran des Chloroplasts besteht aus Proteinen und Lipoiden.

2. Das Stroma ist ebenfalls aus diesen beiden Komponenten zusammengesetzt.

3. Innerhalb des Stromas wurden Membranen mit Oberflächenstruktur beobachtet.

4. Das Stroma enthält ein fibrillares Eiweissgewebe, das wahrscheinlich kein Artefakt ist. Ein ähnliches Gebilde wurde von LEHMANN UND BISS[21] im *Tubifex*-Ei nachgewiesen.

5. Die Granen sind von einer aus Proteinen und Lipoiden zusammengesetzten Membran umgeben. Es ist wahrscheinlich, dass die Lipoide an der Oberflache liegen.

6. Weiter enthalten die Granen Proteinscheiben, welche schon von FREY-WYSSLING UND MÜHLETHALER[9] gezeigt worden sind, und Lipoide.

7. Elektronendiffraktionsdiagramme von isolierten Granen zeigen einen Kreis, welcher auf einen Abstand von ungefähr 2 A hinweist.

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