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STRUCTURE OF PHOTOSYNTHETIC MEMBRANES OF *EUGLENA* USING X-RAY DIFFRACTION

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SUMMARY

Novel X-ray diffraction results of membranes from chloroplasts of *Euglena* are presented, together with freeze-etch images obtained concurrently. Conditions were found for sharp lamellar reflections corresponding to ordered stacking of thylakoids. The periodicity measured by diffraction agrees well with that observed by microscopy. Intensities of diffraction were analysed in order to calculate the electron density distributions across the membranes. Some arguments in favour of the preferred phases of the reflection are given. The distributions indicate firstly the presence of 25 Å-wide regions where the hydrocarbon chains of the membrane lipids are concentrated. This result is discussed in terms of structural models for the chloroplast membrane. Comparison with results of freeze-etching indicates where in the density distribution are the regions inside and outside the membrane sacs. Secondly, the density distributions show maxima on the outside of the membranes only, corresponding possibly to an asymmetrical distribution of lipids.

INTRODUCTION

In the last decade many investigations have been made of the photosynthetic lamellar structures of chloroplasts, using a variety of techniques. Of these electron microscopy has been the most widely employed for structure studies. It has become clear (see for example refs 1 and 2) that the membrane sacs (or "thylakoids") of which the lamellar structures are constituted cannot be adequately described on the basis of the unit membrane idea[3], in particular the membranes seem particulate in nature. The recently developed preparative technique of freeze-etching has played an important part in extending the study of particles in these membranes[4–7]. If the freeze–fracture is considered to be in the hydrophobic membrane "core"[4,6,7] one arrives at a model in which proteins or lipid–protein complexes are embedded in a lipidic matrix which has been described[4,8] to be to some extent "bilayer"-like. It can be seen that these structural models embody several features common to those suggested for many other kinds of membranes (see for example refs 9–13).

In comparison with electron microscopy, the small angle X-ray technique

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has not been widely used as a structural tool for membranes from chloroplasts. As is clear from the description by Kreutz[14], in principle one can obtain important information on the electron density variations both across and in the plane of the membranes, if sufficiently detailed diffraction is recorded. On the basis of several experiments employing the lamellar diffraction (corresponding to density variations across the membranes), which were analysed in a number of different ways[14–16], the thylakoid has been proposed to consist of a sequence of fairly chemically homogeneous lamellae, containing lipids proteins and porphyrin heads. The lipid layer is shown as consisting of two monomolecular layers with the planes containing the polar heads adjacent[11]. In this way the X-ray technique has led to a model rather different from others proposed for the photosynthetic membrane, and from probable structures of other membranes.

This work is aimed at establishing experimental conditions under which some of the difficulties encountered in the application of X-ray diffraction to chloroplasts can be avoided. Our approach has been based on the fact that in *Euglena* chloroplasts[17] groups of up to about five thylakoids are arranged together in discs which extend across the whole width of the chloroplasts (see Fig. 1) without the complex "stack of coins" grana structure so characteristic of higher plants. Consequently we have been able to record sharp Bragg reflections, thus facilitating the X-ray analysis in certain respects, as discussed later. We also consider it advantageous to have simultaneous electron microscopy observations.

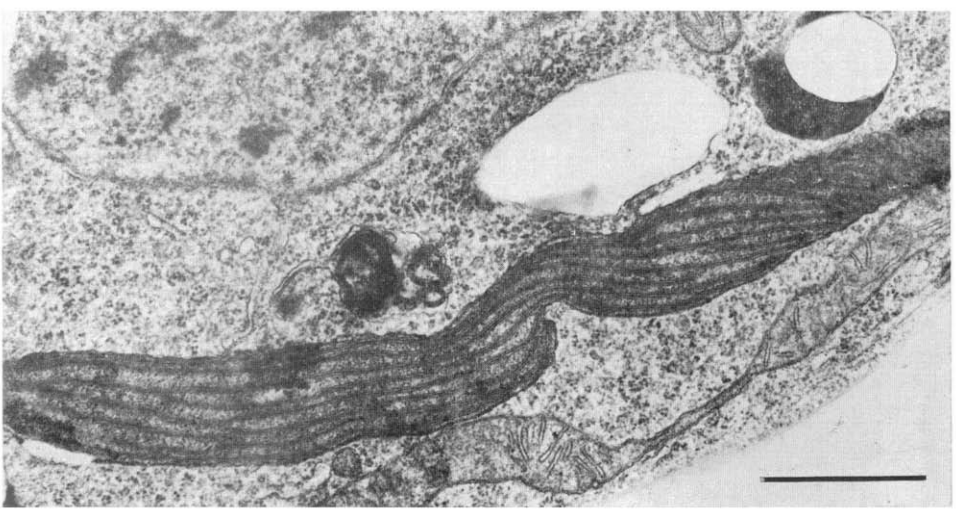

Fig. 1. Section of a chloroplast of *Euglena gracilis* strain Z (glutaraldehyde osmium fixation). Magnification as indicated by the bar of length 1 μm.

EXPERIMENTAL TECHNIQUES

Growth conditions

Euglena gracilis var. *bacillaris* Pringsheim, and alternatively strain Z, were grown heterotrophically on Huntner's medium modified by Greenblatt and Schiff[18]. Harvesting was usually at the end of the logarithmic phase.

Isolation of chloroplasts

The separation of the chloroplasts from the whole organism was effected by the method of Eiseinstadt and Brawerman[19]; in some cases a preliminary flotation was used to remove *Euglena* cells less dense than the average. The medium of extraction contained 10 mM Tris, pH 7.6, 5 mM $MgCl_2$, 0.29 M sucrose, and 1 mM β-mercaptoethanol. All operations, including the diffraction, were carried out with the specimen held near 0 °C.

Microscopy

The chloroplasts were usually resuspended in a medium with the same ionic strength as above, but with the sucrose replaced by 3 M of glycerol. In two cases, the centrifuged chloroplasts (see below) were also studied. A Balzers freeze-etching apparatus and a Hitachi HU 11 electron microscope with a high resolution device were used. Etching was for 75 s at −100 °C.

X-ray procedures

The chloroplasts were centrifuged on to a sheet of mica, which was then transferred to a small sample holder within which the humidity was controlled at about 90%. The X-ray beam was alternatively point or slit collimated; in both cases focussing was with bent glass and the diffraction was recorded photographically. The sample was usually adjusted so that the membranes were tangential to the X-ray beam. Exposure times varied up to 24 h: no significant changes in diffraction were detected within this time.

RESULTS

Microscopy

Images were obtained (Fig. 2) quite in accord with previous freeze-etching studies on chloroplasts of higher plants and green algae[4–7,20]. No evidence of differ-

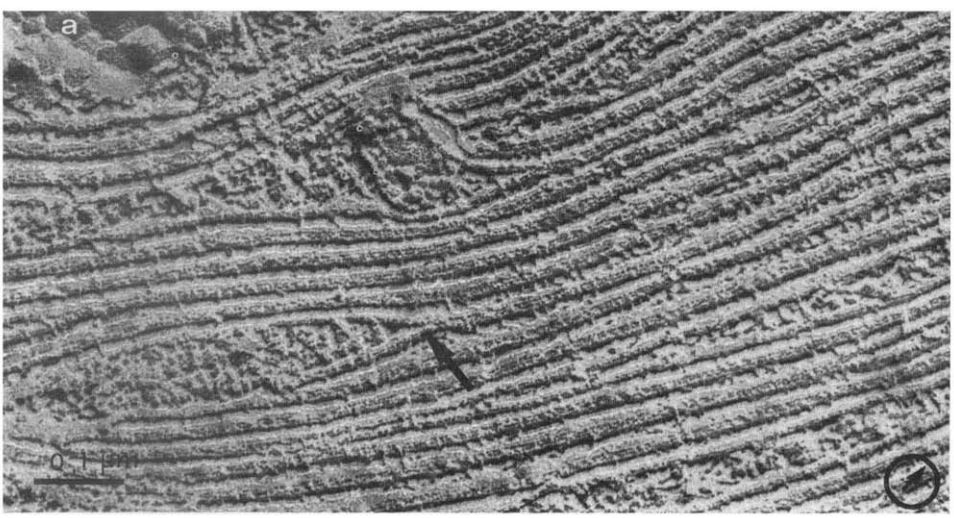

Fig. 2(a).

ences in membrane structure was observed after centrifugation, although the repetition distance in cross-section fractures is reduced. Nor were any differences observed after storage of the specimen. After centrifugation the cross-sectional repeat distance was about 180 Å. Between fairly deep furrows can be seen much shallower troughs: these differences in depth must correspond to differing extents of sublimation. Although thylakoids in *Euglena* are usually in the same disc for the entire width of the chloroplast[17], occasionally they separate from each other, as shown by the arrow in Fig. 2a. It can be seen that the plane of separation is

Fig. 2(a). Freeze-etch image of chloroplast material from *Euglena gracilis* var. *bacillaris* in a buffer containing 3 M glycerol. An arrow indicates where two thylakoids come together.

Fig. 2(b). Material similar to that in (a), but after centrifugation on to mica in 1 M glycerol. In regions fractured along the surfaces there is more than one kind of particulate structure, as reported already for other sorts of chloroplasts. An arrow indicates lamellae of thickness about 70 Å.

continuous with a furrow which is little sublimed, which is therefore along the plane separating thylakoids. The inside of the sacs then corresponds to the wider sublimed regions. We assume the freeze–fracture to take place in the hydrophobic zone as proposed by Branton and Park[4] (*cf.* Kreutz, ref. 14, p. 137). It was observed[4] that the distance between cleavage planes is greater within the thylakoid than it is between adjacent thylakoids. This is in agreement with our identification of the wider of the sublimed zones as the thylakoid interior. Occasionally, lamellae of thickness about 70 Å could be seen (Fig. 2 b). As will be clear also from the diffraction results, the material contains more than one kind of structural entity.

X-ray diffraction

The following generalisations summarise results from more than 15 separate chloroplast preparations. The diffraction depended critically on the humidity: if this was greater than about 90%, there were two diffuse small angle bands between angles corresponding to 0.011 $Å^{-1}$ and 0.017 $Å^{-1}$ and also between 0.025 and 0.033 $Å^{-1}$. In other words, there is a minimum in diffuse scatter at an equivalent Bragg spacing of 47 Å. At about 90% humidity about six sharp oriented reflections

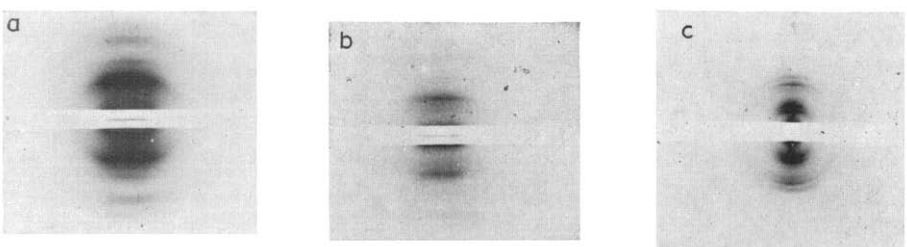

Fig. 3. (a and b) Diffraction of centrifuged chloroplasts of *Euglena gracilis* var. *bacillaris*, using slit collimation (two exposures are illustrated). (c) Diffraction of material similar to that for (a), using point collimation. The diffraction is intensified along a line perpendicular to the chloroplast mat.

could be observed (Fig. 3). Humidity much less than 90% resulted in a small number of broad reflections. Variations, examples of which are given below, in the diffraction observed for a large number of samples enabled four of the six reflections to be identified as the second, third, fourth, and fifth orders of diffraction of a lamellar system of periodicity near to 170 Å. Fig. 4 shows a microdensitometer trace and the identification of the maxima corresponding to a repeat distance of 167 Å. The table shows intensity values for four examples of the sort shown in Fig. 4 (the three periodicities near 165 Å cannot be considered to differ significantly). The intensities are corrected using a factor of n^2 where n is the order of diffraction.

As is clear from Fig. 4, there are two reflections which are not indexed as indicated in the table. These two reflections are very variable in intensity, and also to some extent in position, relative to those corresponding to the 170 Å repeat. These variations arise as a consequence of different chloroplast preparations using different cultures of *Euglena*, and also of variations in the precise conditions of drying. Since both "supplementary" reflections show orientation (Fig. 3) they also arise from lamellar structures. Chromatography[21] of the lipid extracted from

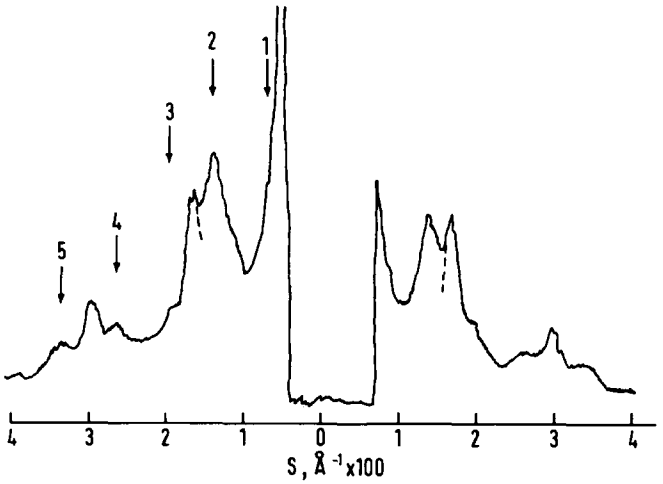

Fig. 4. Microdensitometer trace of a film similar to that shown in Fig. 3c, but less exposed. The identification of the orders of the thylakoid diffraction is indicated by numbers; the first order is not detectable. This example shows clearly the resolution of the diffraction peaks, and shows a large contribution to the diffraction which cannot be attributed to the thylakoids (see text). S is a measure of the diffraction angle, expressed in $Å^{-1}$. The ordinate indicates absorbance (A).

some of our preliminary chloroplast preparations, for which the reflection at 0.0263 $Å^{-1}$ (a Bragg spacing of 38 Å) was particularly strong, suggests some degradation of the galactolipids. In addition, it is known[17] that globules, very likely of lipid, exist in *Euglena* chloroplasts. In this way circumstantial evidence suggests that the reflection at 0.0263 $Å^{-1}$ can be attributed to lipid leaflets separate from the thylakoids. At present there is no unambiguous identification of the other supplementary reflection, near 0.015 $Å^{-1}$. This can however be very weak (Fig. 3b), and it is clear on the basis of its width alone (Fig. 4) that it cannot be indexed simply together with the other reflections.

Trial experiments with the X-ray beam perpendicular to the chloroplast mat suggest that there are structures within the plane of the lamellae giving rise to a broad reflection at 85 $Å^{-1}$. This may correspond to the sort of in-plane diffraction upon which much of the previous X-ray work has been based[14].

INTERPRETATION AND DISCUSSION

The agreement between X-rays and microscopy is excellent as to the dimensions of the stacks of double membranes. The intensity measurements (Table I) can now be used to study the electron density distribution across the thylakoid.

The choice of phases for the reflections, necessary for the calculation, is the one that is the most probable on the basis of the X-ray results (see below). Another criterion in favour of our choice of phases is that it enables the X-ray and freeze-etching results to be interpreted in a way which is consistent with the current concepts of membrane structure as embodied in previously proposed structures for photo-

TABLE I

RELATIVE INTENSITIES

n is the order of diffraction.

Periodicity of thylakoids (Å)	*Relative intensities*				
	n= 1	*n*= 2	*n*= 3	*n*= 4	*n*= 5
167	<0.1	16	1.3	3.3	10
164	<0.1	17	2.6	2.8	7.8
165	<0.15	18	2.6	4	6
174	<0.4	9.7	5	3.7	10.5

synthetic and other membranes.[4,8–13] Fig. 5a shows a representative example of an electron density profile, corresponding to a periodicity of 165 Å, calculated with the phases 0, 0, π and π for the second, third, fourth, and fifth orders of diffraction, respectively. Two arguments based on the X-ray data which are in favour of this choice of phases are as follows. The change of phase between the third and fourth orders is consistent with the mimimum in diffuse scatter observed with humidities higher than 90% at the corresponding angle, *i.e.* at 0.021 $Å^{-1}$. This supposes that at high humidities the lamellae swell slightly in the region corresponding to 80 Å

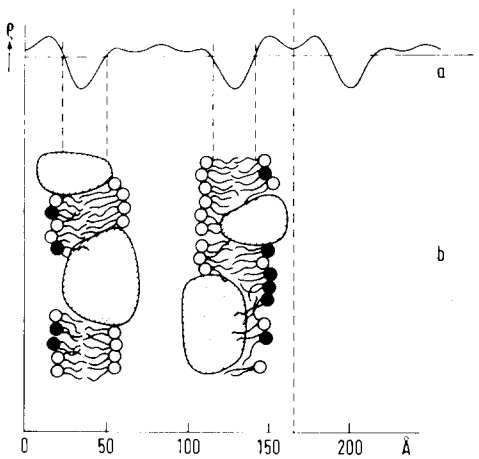

Fig. 5. (a) An example of the proposed electron density projected on to a line perpendicular to the thylakoids, calculated using the third set of data in the table. ϱ is electron density relative to the average electron density and is expressed on an arbitrary scale. (b) An illustrative diagram showing the sort of structural model for the thylakoid membranes which would be most consistent with the X-ray and freeze-etching results. Comparison of X-ray and electron microscopy results enables an identification of the double membrane as shown here with one thylakoid, *i.e.* the region between the membranes is the interior of the sac. Previous authors have suggested very similar structures (see text), and discuss more fully the particle asymmetry across each membrane. The lipid molecules are indicated in the conventional manner, their head groups being shown as circles. The possibility of lipid asymmetry is indicated by the head group circles being shaded in two ways. The diagram is not drawn to scale in the direction parallel to the membranes.

on the abscissa of Fig. 5a and are then not stacked in a very perfect way. This explanation of the diffuse scatter may not be entirely adequate since there are other diffracting structures present. Secondly, profiles calculated from the four sets of data using this combination of phases differ slightly in the width of the approximately flat region centred at 80 Å on the abscissa, but differ little in the rest of the profile.

The calculated electron density distribution shows two regions about 25 Å wide of density markedly different from the average: Fig. 5a illustrates these as being of low density. The centres of these regions are spaced alternately by 72 Å and 93 Å throughout the structure. We identify them with the two membranes of the thylakoids where one can expect a concentration of the electron-light hydrocarbon chains of the lipids. The width of these two regions is similar to the width of the hydrocarbon layer as found in model systems of lipid and water[22] or lipid water and protein (Sadler, D. M. and Gulik-Krzywicki, T., unpublished). Thus Fig. 5a suggests the presence of hydrocarbon zones of a width defined by the length of two opposed lipid chains. It should be emphasised that the absence of a measure of the depth of the hydrocarbon minimum means that it is in no way excluded that some proteins, for example, would be in the same plane as the hydrocarbon. Our ignorance at the moment of the relative concentrations of lipid protein and water in the diffracting structure precludes an estimate of how much protein could be in the hydrocarbon layer, in the way that is possible for more completely chemically defined systems[11,23,24].

On freeze-fractured surfaces one sees smooth zones between particles: an example of the way one can make a synthesis of the X-ray and freeze-etching results would be to propose regions of lipid lamellae (with the hydrocarbon chains in contact) interspersed with proteins or lipoprotein particles. This scheme is illustrated in Fig. 5b. The extent to which the particles penetrate the hydrocarbon zone, and their structure, is not defined on the basis of the techniques discussed here. The two regions within each repeat unit, of relatively higher density, and which separate the hydrocarbon layers, are likely to contain significant amounts of water. It will be noted from Fig. 5a that one of these regions is wider than the other. Water will sublime after freeze–fracturing: in this way we can identify the wider of the water-containing parts of the structure with the larger of the two furrows seen on the transverse fracture surfaces. The latter have already been deduced to be at the thylakoid interior (see above). Therefore 80 Å and 245 Å on the abscissa of Fig. 5 correspond to the centres of the thylakoids, and 0 Å and 165 Å to the planes of contact between thylakoids.

Comparison of the Figs 2a and 2b shows that it is the width of the larger of the sublimed zones, identified here with the thylakoid interior, which can be varied by the experimental conditions (in this case by centrifugation). Likewise, the X-ray analysis has indicated that it is the region centred at 80 Å (Fig. 5) which can be varied in width. The junction between each thylakoid in a disc is analogous[17] to the distinctive zone of relatively well-defined width[8] which is known as the partition in the case of the grana of higher plants (see for example ref. 1). It is likely that the ordered one-dimensional stack of thylakoids, which is giving rise to the observed diffraction, is one in which this partition-like structure of well defined width exists between most thylakoids. It is then entirely reasonable, as implied by

the above analysis, that the distance between membranes of different thylakoids is shorter and less variable than the distance between membranes of the same thylakoid.

It will be clear that there are significant differences between this and some previous interpretations, in particular those based on previous X-ray results[14]. The differences between Fig. 5a and previously proposed electron density distributions may be due to the different source of material; differences in experimental data can be judged by a comparison of Fig. 4 and Table I with Fig. 9 of ref. 25 and with Figs 17, 22 and 23 of ref. 14. Several other differences could well occur however since in the present case the analysis is carried out in terms of the intensity of sharp Bragg maxima. As a first consequence, one is able to distinguish between diffraction from thylakoids and from other entities which must be supposed to be present, in *Euglena* chloroplasts at least. Secondly, the phasing procedure is different; recent calculations of Kreutz[14] overcome this problem by a deconvolution of the "Q-function". In doing so one relies heavily on the detailed variation of diffraction with angle, which may not be justified in view of possible sample inhomogeneity and other experimental difficulties. Earlier analyses[15,16] were dependent on an *a priori* choice of model. The phasing proposed in the present work would follow from an *a priori* choice of a model of the general type as shown in Fig 5b[4,8–13]; this can be done by inspecting all the Fourier transforms, using all possible permutations of phases. In addition however there are two independent arguments which favour the phasing adopted as being the most probable, based on the diffuse scatter at high humidities and on slight differences between separate experiments. The analysis as a whole also implies, very reasonably, that the inter-thylakoid membrane separation is less, and less liable to variation, than the intra-thylakoid separation. The disparity between Fig. 5b and the conclusions of Kreutz may also arise from the identification of molecular positions in terms of electron density, which is not necessarily unambiguous. Indeed, Kirk[8] has proposed a structure, essentially the same as shown in Fig. 5b, which is argued to be fully compatible with the electron density distribution proposed by Kreutz.

The distribution (Fig. 5a), in addition to its relevance to the foregoing discussion, contains other information on the membrane structure. There is a thylakoid asymmetry because the region inside the thylakoid is larger than that outside. In addition, however, Fig. 5a shows an asymmetry across each of the membranes, which is much more pronounced than for other membranes studied in detail by X-rays[10,11]; compare Fig. 5a with, for example, the profile derived for retinal disc membranes using diffraction data to a similar resolution[11]. The asymmetry is probably significant since it is very pronounced on all the profiles derived from the four sets of data. There is no comprehensive information available concerning the electron density of the various chloroplast components, but it seems possible that the electron-heavy head groups of the sulpholipids and of phosphatidyl glycerol could contribute to the high density on the outside of the membrane. (The polar heads of the galactolipids, the major lipid component, will be expected to be less dense.) The resolution is however not sufficient to image clearly a plane of ionic head groups, if such a localisation of head groups obtains. The idea of the asymmetry of the thylakoid membranes has often been discussed, especially from a functional point of view[6,26]. Evidence for structural asymmetry exists in that the

particle distributions vary according to the face of the membrane which is imaged using freeze-etching[4–7,20]. The present X-ray results provide an indication that the molecular structure is different on each side of the membrane.

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