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ON THE MOLECULAR NATURE OF CHLOROPLAST THYLAKOID MEMBRANES

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SUMMARY

Envelope- and stroma-free thylakoid membranes of *Vicia faba* chloroplasts were disintegrated and the electrophoretic behavior of the components studied with special regard to the pigment-protein complexes. The process of denaturation of the complexes was found to differ with respect to the other protein components. As the result of denaturation, the pigment-free protein moieties exhibit altered electrophoretic mobilities in relation to the "intact" complexes mainly conditioned by two processes contrary in their action, i.e. increase of charge and change of the hydrodynamic properties.

Exhaustive extraction of the thylakoid membranes with 6 M guanidine · HCl removes the proteins mainly associated by polar and weak hydrophobic interactions. The insoluble residue quantitatively exhibits the pigment-protein complexes including their denatured protein moieties, two extrinsic hydrophobic proteins as well as some protein traces. Electron-microscopic studies demonstrate the material still to have a high degree of order and preserved basic structure. After removing the lipids from the basic membrane, large amounts of the protein moiety of Complex II become soluble in guanidine · HCl. Since all other lamellar proteins are removable either by guanidine · HCl extraction or by trypsin digestion it is assumed the basic membrane of thylakoid to consist only of the pigment-protein complexes embedded into a lipid matrix.

INTRODUCTION

The conceptions of the molecular structure of chloroplasts presently discussed are based mainly upon electron-microscopic data and X-ray diffraction studies. The

Abbreviations: Complex I and II, pigment-protein complexes of Photosystems I and II, respectively; Complex I protein and Complex II protein, protein moieties of Complex I and II, respectively.

results obtained have contributed substantially to the creation of models of the organization of the thylakoid membranes, although considerable disagreement exists as to how the chemical components of the membranes are assembled together to give the photosynthetically functional apparatus. Apart from the remarkable attempt of Kirk [1, 2] to reconcile differing conceptions, the molecular construction of thylakoid membranes is still a point of controversy. Therefore, efforts have been made to find out whether or not further information can be obtained by the application of chemical methods. The aim of the experiments was to gather additional data for a model describing the native state of thylakoid membranes with as much precision as possible.

MATERIALS AND METHODS

Isolation and purification of stroma-free thylakoid membranes

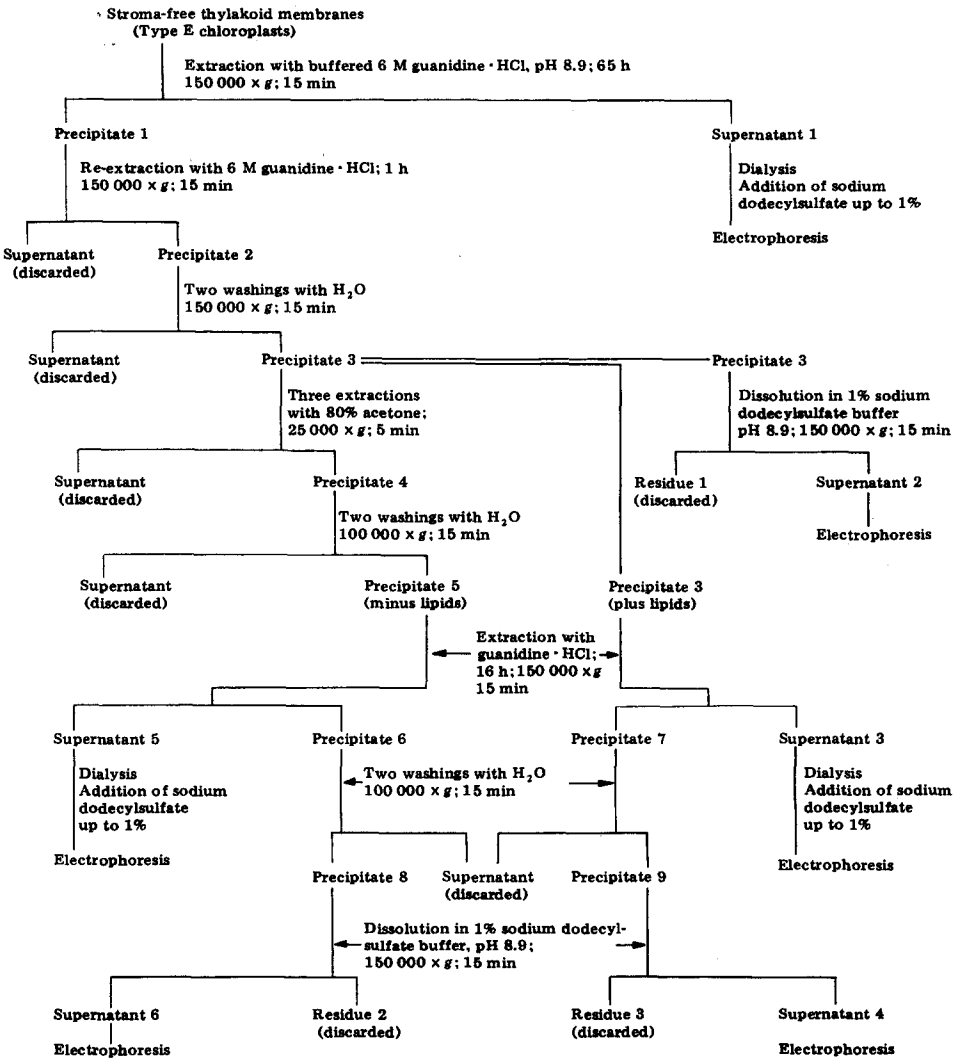
Plants of *Vicia faba* grown in a modified Hoagland solution were cultivated in a phytotron for 6 weeks. Chloroplasts of leaves harvested immediately prior to being homogenized were isolated according to the flow sheet published previously [3]. The material was purified by density-gradient centrifugation using the method of James and Das [4]. The envelopes of chloroplasts enduring this procedure were burst open by hypotonic shock in water. After separation of the envelopes by centrifugation, the attached stroma proteins were removed by extensive extraction with water. The resulting material corresponds to the Type E chloroplasts of Hall [5] and exhibits swollen thylakoid membranes as shown in Fig. 1. All operations were carried out at +2 °C.

Extraction of associated proteins

Proteins extrinsically attached to the membrane surfaces were removed according to the following flow sheet by continuous stirring of the material in a dark coldroom at +2 °C with 6 M guanidine · HCl dissolved in 0.03 M sodium borate/HCl buffer, pH 8.9. Since sodium dodecylsulfate precipitates at low temperatures, all operations with this denaturant were carried out at room temperature. To prevent precipitation between guanidine · HCl and sodium dodecylsulfate molecules it was necessary to remove guanidine · HCl by extensive washings with water before starting sodium dodecylsulfate extraction. In all experiments, chlorophyll was found to be present quantitatively in the guanidine · HCl-insoluble residue. Since the distribution of chlorophyll to the different lamellar components has been described previously [6], chlorophyll values are not given in the present paper. To remove bivalent cations, the thylakoid membranes were extracted with 1 mM EDTA, pH 8.9, for 1 h. Extracts were concentrated and dialysed using an Amicon micro-ultrafiltration system 8 MC.

Gel electrophoresis

Sodium dodecylsulfate up to 1 % was added to all extracts before electrophoresis to keep to same conditions in all experiments. Gel electrophoresis and molecular-weight analysis were carried out as published previously [3, 7, 8]. To obtain comparable results, the columns (5.8 mm diameter) were loaded with 40 μg protein in all cases. The stained gels were scanned with a Chromoscan densitometer of Joyce and Loebl using a 595 nm filter.



RESULTS

Protein patterns of the entire thylakoid membranes

Envelope- and stroma-free thylakoid membranes (Fig. 1) isolated and purified according to the procedure described, and disintegrated by sodium dodecylsulfate exhibit a protein pattern* shown in Fig. 2a after electrophoretic separation. Long-term re-extraction with water results in no fundamental alteration of the pattern and, therefore, it may be assumed that all those proteins are either associated

* The protein patterns were denoted as published previously [7, 8], although, as a result of improving the electrophoretic procedure, more bands appeared in comparison with former experiments.

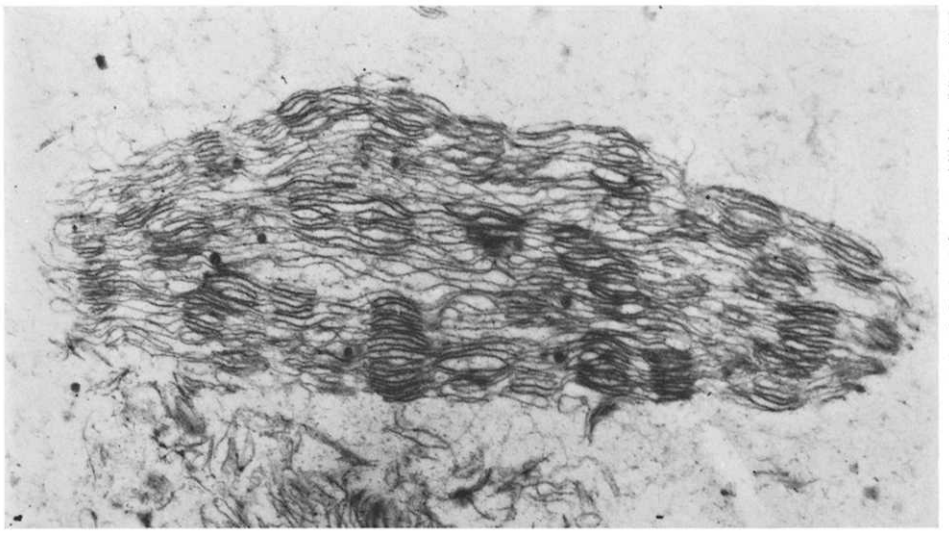


Fig. 1. Envelope- and stroma-free thylakoid membranes of *Vicia faba* chloroplasts swollen by hypotonic shock. Fixed in 3 % osmium tetroxide.

with the membrane or are an intrinsic part of it. According to the action of the detergent, the character of the binding forces between the different membrane components in the native state must be non-covalent and the bonds will be treated together as interaction between polar groups (hydrogen bonds) or between non-polar groups (hydrophobic bonds) [9].

After removing the pigments by higher concentrations of sodium dodecylsulfate or by acetone, the protein moieties of the pigment-protein complexes exhibit altered electrophoretic mobilities (Fig. 2b) as compared with the "intact" (undenatured) complexes in which most of the pigment-protein bonds are being preserved. The protein moiety of Complex I migrates significantly faster than the "intact" complex. In contrast, the protein moiety of Complex II after extraction of the pigments has a somewhat lower mobility, compared with the "intact" complex. Although the difference is rather small, densitometric studies demonstrate that the effect obtained within a range of acrylamide concentration of 9–10 % is significant. The cause of the different behavior of the two protein moieties is not yet exactly known and more informations on the chemical nature of the proteins are necessary to understand the connections. According to the studies of Reynolds and Tanford [10] it can be concluded that two main processes, contrary in their action, may affect the electrophoretic mobility of sodium dodecylsulfate-protein complexes; increase in electrostatic charge per unit mass by the anionic head groups of the detergent molecules force the protein to move faster whilst the transformation of globules into rodlike particles alters the hydrodynamic properties of the complexes and, consequently, electrophoretic mobility tends to decrease. It can be assumed that the two processes influence the electrophoretic mobilities of the protein moieties of the pigment-protein complexes differently.

Removal of pigments by acetone, as well as heating of the detergent-protein extract or addition of reducing agents, increases the tendency of Complex I protein to

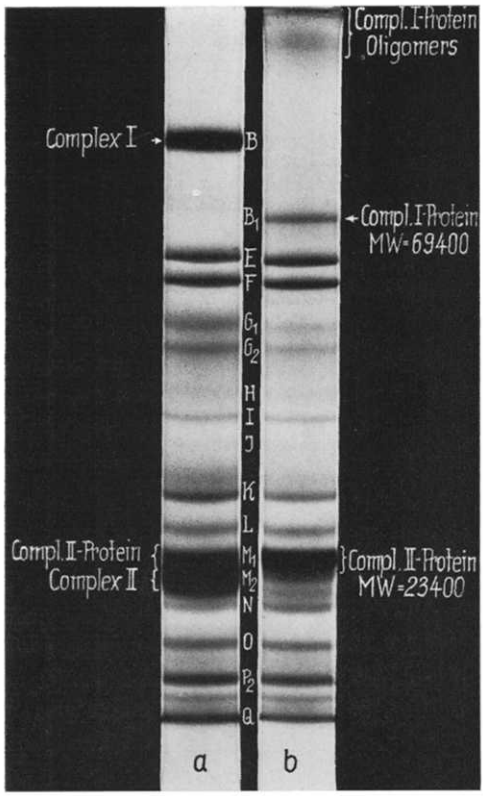


Fig. 2. Protein patterns of thylakoid membranes disintegrated with sodium dodecylsulfate buffer and separated by polyacrylamide gel electrophoresis. (a) Protein pattern of membranes without removing lipids. (b) Protein pattern of membranes after extraction of lipids with 80 % acetone. Removing the lipids alter the electrophoretic mobility of the protein moieties of the pigment-protein complexes.

form oligomers (Fig. 2b). The process is not really understood: however, re-electrophoresis with pure Complex I obtained after preparative separation and following elution demonstrate the effect to be significant.

The experimental results support earlier conclusions, based upon analytical results, with respect to the amino acid composition of the pigment-protein complexes (11) and indicate essential differences in the structure of the native complexes as well as in the chemical nature of the protein moieties. Furthermore, the different electrophoretic behavior of the protein moieties in relation to the "intact" complexes should be kept in mind if the question has to be answered of whether or not the protein moieties of the complexes are present in defective chloroplasts.

Associated proteins and basic membrane

Detergents are known to disrupt hydrophobic chemical bonds up to rather strong binding forces. So far, they differ at least quantitatively from other denaturants, e.g. urea and guanidine · HCl. Although the action of these substances is not exactly understood, it can be concluded from data of the literature [12–15] that urea and

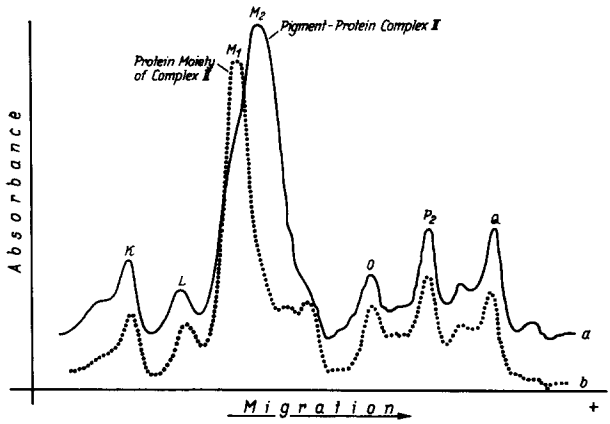


Fig. 3. Densitogram of pigment-protein Complex II. In contrast to Complex I the denatured and chlorophyll-free protein moiety of Complex II (b) migrates slower in relation to the "intact" complex (a). The figure represents a section of Gel 2b.

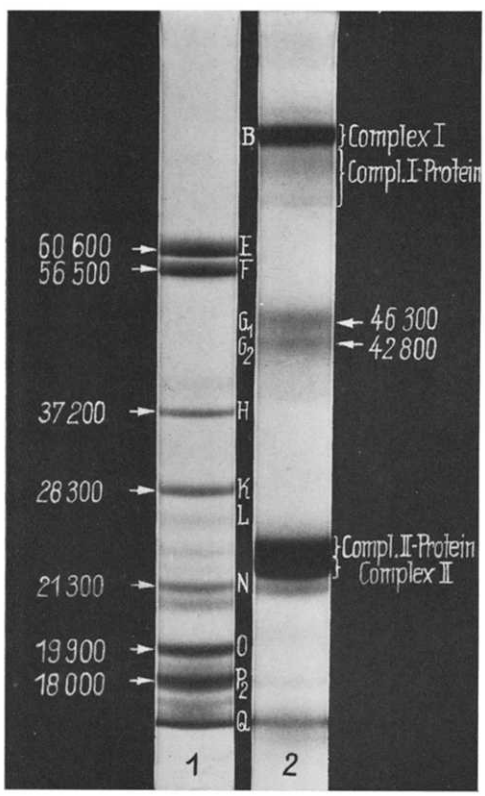


Fig. 4. Patterns of associated proteins (1) and basic membrane proteins (2). Treatment of thylakoid membranes with 6 M guanidine · HCl removes the protein associated with the basic membrane mainly by polar and weak hydrophobic interactions. Gel 1 represents Supernatant 1 and Gel 2, Supernatant 2, respectively.

guanidine · HCl are able to dissolve mainly polar bonds and weak hydrophobic bonds. Concerning the thylakoid membranes, it can be expected that treatment with urea and guanidine · HCl removes primarily compounds which are attached to the membrane surface, while intrinsic components of the lipid-protein structures are insoluble.

Starting from these considerations, 6 M guanidine · HCl was applied to remove the so-called associated proteins from their binding sites. As shown in Fig. 4, the protein pattern of the guanidine · HCl extract (Supernatant 1) exhibits most of the lamellar components. Densitometric studies point out 40–45 % of the protein content of the stroma-free thylakoid membranes is being soluble under these conditions. After dissolution in sodium dodecylsulfate buffer, the guanidine · HCl-insoluble material (Precipitate 3) contains the following components: the pigment-protein complexes and the corresponding protein moieties, two hydrophobic proteins denoted as G_1 and G_2 and small amounts of proteins corresponding with Bands N and Q. Further extensive extraction according to the flow sheet results in four main components (and some traces of other proteins) which are constituents of the guanidine · HCl-insoluble material (see Gel No. 4 in Fig. 7, representing Supernatant 4). According to recent experiments of Süss and Schmidt (unpublished) the G-proteins, although insoluble with guanidine · HCl, are digestable with trypsin very easily, demonstrating these proteins also to be extrinsic but, in contrast to the other associated proteins, bound to the basic material by strong hydrophobic interactions. Electron-microscopic studies of Precipitate 3 show the material still to have a rather high degree of order with the basic structure of the thylakoid membranes preserved (Fig. 5). Since, with the exception of small amounts of starch (Residue 1), Precipitate 3 can be dissolved almost quantitatively with the applied sodium dodecylsulfate

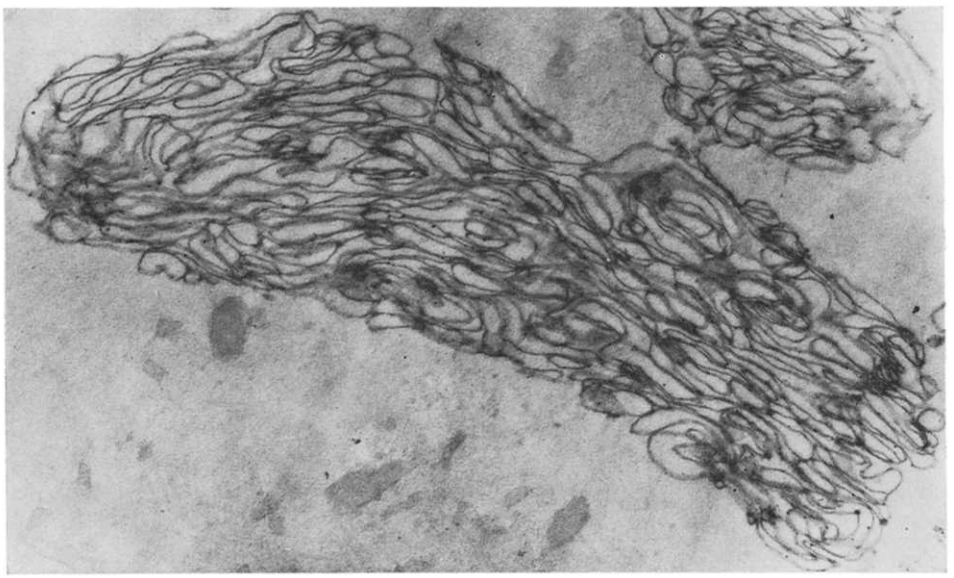


Fig. 5. Electron-microscopic interpretation of the basic membrane of *Vicia faba* thylakoids. The material represents Precipitate 3 and demonstrates the basic membranes after removing the associated protein with guanidine · HCl. Fixed in 3 % osmium tetroxide.

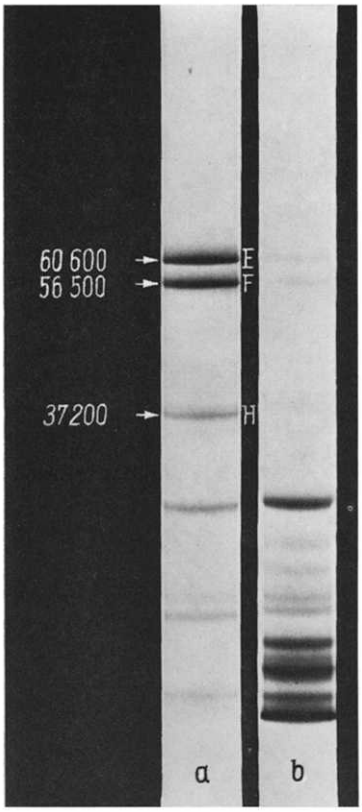


Fig. 6. Fractionation of associated proteins by extracting the thylakoid membranes with 1 mM EDTA (a) followed by re-extraction of the same material with 6 M guanidine · HCl (b).

buffer, the protein pattern obtained with Supernatant 2 represents the molecular composition of this material.

The results support the theory that the backbone of the thylakoid is a rather stable construction and that the denotation "basic membrane" is justified. Furthermore, the present findings and the unpublished results obtained by trypsin digestion indicate the basic membrane to consist only of lipids and the two pigment-protein complexes. As long as lipids are present, the two complexes are insoluble in guanidine · HCl and rather resistant to trypsin digestion.

With respect to the associated proteins, most of these components can be assumed to be connected with electron transport and photophosphorylation. Although the protein bands representing the pattern of Gel 1 (Fig. 4) are not yet exactly characterized it seems to be certain that the compounds differ not only in their function but also in their mode of interaction with the basic membrane. It is well established that some of the proteins are nearly quantitatively extractable with 1 mM EDTA, whilst other proteins need high concentrations of guanidine · HCl (Fig. 6). Efforts are being made to characterize the associated proteins. According to preliminary results based upon molecular weight determination the Bands E, F and H very probably represent subunits of photophosphorylase.

Experiments on the construction of the basic membrane

The resistance of the basic membrane to protein denaturants of the urea and guanidine · HCl type raises a question as to the molecular arrangement of the different chemical components which form structures of high stability. To find an answer, basic membranes (Precipitate 3) were extracted with 80 % acetone to remove most of the lipids [16]. The residue (Precipitate 5) was re-extracted with 6 M guanidine · HCl to find out whether or not basic membrane proteins, after removing the lipids, can be solubilized by this type of protein solvents. The extract (Supernatant 5) was dialysed and, after addition of sodium dodecylsulfate, the electrophoretic pattern of the proteins was compared with the pattern of the guanidine · HCl-insoluble residue disintegrated with sodium dodecylsulfate buffer (Supernatant 6). In addition, basic membranes without removing the lipids but treated in the same manner as described (Supernatants 3 and 4), were incorporated into these experiments to have a reference base.

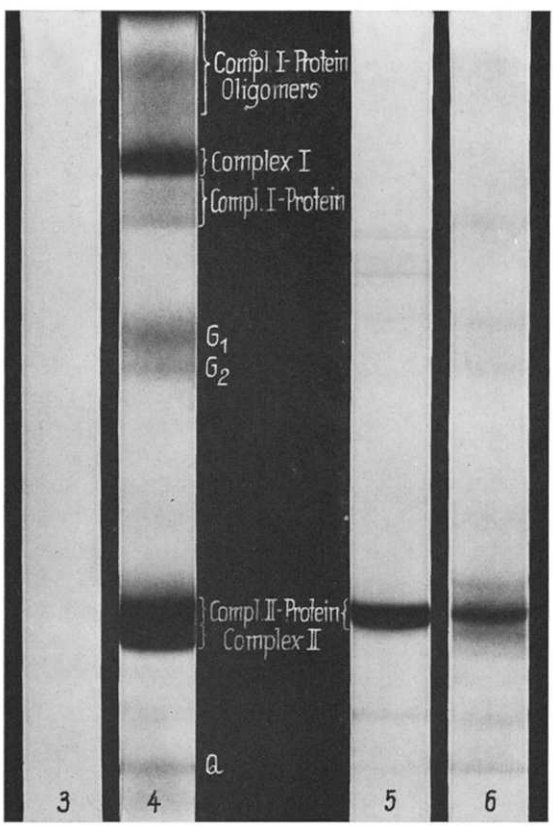


Fig. 7. Influence of lipids on the stability of basic membranes to guanidine · HCl. Basic membrane proteins are insoluble in guanidine · HCl as long as lipids are present (Gels 3 and 4 represent Supernatants 3 and 4). Removing most of the lipids by extraction of the basic membranes with 80 % acetone exhibits large amounts of the protein moiety of Complex II now being extractable by the action of guanidine · HCl (Gels 5 and 6 represent Supernatants 5 and 6).

The results concerning Complex II protein are represented in Fig. 7 (Gels 5 and 6). They exhibit large amounts of polypeptides which are not extractable if lipids are present but can be solubilized by the action of guanidine · HCl after lipid removal. This behavior of Complex II protein seems to be understandable only if it is assumed that rather large regions of the polypeptides are composed of polar amino acids. Contrary to this, only traces of Complex I protein are present in the same gel, indicating that the character of this compound is more apolar in relation to Complex II protein. Beyond this, other chemical reactions, presently not exactly understood, must occur to form insoluble aggregates since Complex I protein also cannot be solubilized by subsequent extraction of Precipitate 8 with sodium dodecylsulfate buffer.

The remarkable decrease of the resistance of Complex II protein to guanidine · HCl after removing the lipids shows these compounds to play an important role with regard to the stability of the intact complex in a hydrophilic surrounding. On the basis of these findings it may be suggested that the pigment-protein complexes are inserted into a lipid matrix.

DISCUSSION

In evaluation of the protein patterns represented in Figs 2, 4, 6 and 7, the native state of the proteins in living matter should be kept in mind. It is well established that many of the proteins consist of two or more subunits exhibiting a specific tertiary structure and constructed from one or more covalently bonded polypeptide chains. The subunits are assembled together by non-covalent interactions to form the quarternary structure of the native proteins.

For the components of thylakoid membranes, similar conformational structures should be expected. Photophosphorylase, for instance, is supposed to consist of five subunits [17, 18]. The native pigment-protein complexes of higher plants, which are believed to originate in Photosystems I and II, respectively, may also be assumed to be constructed from subunits. The water-soluble bacterial chlorophyll protein was found to have a molecular weight of 152 000 and to consist of four identical subunits [19]. Results similar in tendency were obtained with higher plants, although considerable oscillations exist with respect to the molecular weight of the native complexes [20]. Considering the results of the present paper under these aspects, the protein bands obtained after sodium dodecylsulfate disintegration of the membranes and electrophoretic separation of the components represent mainly subunits.

Other findings are being supported with respect to the proteins attached to the membrane surface [15, 21]. The present results indicate that most of the lamellar proteins are associated by polar or weak hydrophobic interactions. It seems to be evident that only the pigment-protein complexes of the basic membrane are intrinsic membrane components and possibly correspond to "structural" [22] or "bimodal" [23] proteins. At least parts of proteins represented in Bands N and Q may also be membrane-intrinsic. Finally, it can be assumed the completed, photosynthetically functional thylakoid may be the result of association of functional proteins and non-protein components with the basic membrane.

So far, the present results correspond with the definition of the basic membrane

given by Kreutz [24]. Yet, concerning the arrangement of the protein components within the basic membrane, it is doubtful whether or not the three-layer model [24–26] can explain the native state. According to this theory, structural-protein crystallites are arranged in a distinct layer with a high degree of order and connected with a distinct lipid monolayer by porphyrin-lipid interactions. Although the question of the molecular architecture must still be regarded as open, recent conceptions tend more and more to postulate membranes to be constructed, possibly by a self-assembling process [1], of globular proteins embedded into a lipid matrix which is believed to be the structural framework of the membrane [27]. The conclusions drawn in the present paper on the basis of the obtained experimental results correspond with these considerations and, beyond it, support the conceptions of other authors [2, 28, 29.] Conformity may also exist with the binary membrane model of thylakoids [30, 31].

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