

The Correlation of Lipid Release and Photochemical Activities in Isolated Spinach Chloroplasts

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Purified isolated spinach chloroplasts were suspended in media of different pH and the extracts obtained after centrifugation were analyzed for their lipid composition. The quantity of lipids present in the supernatant increased considerably by lowering the proton concentration. Under alkaline conditions more than 30% of the phospho- and sulfolipids were released from the thylakoid membranes. A stabilization was achieved when higher concentrations of $MgCl_2$ were present. Photochemical activities showed an uncoupling of photophosphorylation at pH 8.5 and 8.9, and a stabilization at pH 5.5. The correlation between phosphorylation and the release of lipids was further demonstrated in the presence of $MgCl_2$ when the ATP-formation was protected under alkaline conditions. From the comparison of the lipid composition in the extracts obtained from broken chloroplasts and from fragments after sonication, the conclusion can be drawn that part of the phospho- and sulfolipids and monogalactolipids are located at the surface of the membrane. The majority of lipids released are bound to particles as demonstrated by the sedimentation in the ultracentrifuge.

The molecular composition of chloroplast membranes embedding the photosynthetic machinery has been described in reports from several laboratories^{1–3}. Major representatives of the structural and functional components are the lipids. From several analyses, acyl lipids were shown to be present in high quantities. However, their localization in the thylakoid membrane, their orientation and participation in functional events are still open problems⁴.

From X-ray diffraction studies KREUTZ and MENKE⁵ and later KREUTZ⁶ postulated that the lipids are located in the inner layer of the thylakoid membrane. Recently, KREUTZ⁶ developed a model in which

the phospholipids are involved in the charge separation during the formation of the proton gradient. The amphoteric groups were discussed as being arranged vertically across the lipid layer. Studies with antibodies against sulfoquinovosyl diacyl glycerol and phosphatidyl glycerol suggested that these polar lipids are also inside the thylakoid^{7,8}. However, as pointed out by RADUNZ and BERZBORN⁸, some sulfoquinovosyl residues seem to be oriented against the outer protein layer.

An entirely different concept was developed by WEIER and BENSON⁹ and recently more pronounced by BENSON *et al.*¹⁰. From a chemical point of view they suggest a membrane composed of protein subunits and associated with surfactant lipids. That lipids are located in the outer part of the thylakoids has also been concluded from the results of LIVNE and RACKER¹¹ who demonstrated the protection of the coupling factor involved in photophosphorylation against heat inactivation, especially by sulfolipids. The coupling factor was already shown to be located at the surface of the thylakoids and could easily be removed by EDTA^{12–14}. Consequently, if the polar lipids are constituents of the phosphorylating system, either involved in an architectural orientation or even more directly in the mechanism of ATP-formation, at least some of these compounds should be localized at the surface of the thylakoid membrane.

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Abbreviations: ADP, Adenosinediphosphate; ATP, adenosinetriphosphate; BSA, bovine serum albumin; DEGS, diethyleneglycolsuccinate; DGD, digalactosyl diglyceride; DGD-X, derivative from Digalactosyl diglyceride; EDTA, ethylenediaminetetraacetate; MES, 2-(N-morpholino) ethane-sulfonic acid; MGD, monogalactosyl diglyceride; MGD-X, derivative from monogalactosyl diglyceride; NADP⁺, nicotinamide adenine dinucleotide phosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; Pi, inorganic phosphate; PS-I(II), photosystem-I(II); SL, sulfoquinovosyl/diglyceride; Tricine, N-Tris-(hydroxymethyl)-methylglycine.



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The correlation of lipid composition and photochemical activity through direct chemical estimations is still lacking. The decrease of photochemical activities observed after longer storage of isolated chloroplasts was suggested to be correlated with lipid transformations¹⁵. The secondary effects were different and depended upon the composition of the media used for the isolation of chloroplasts^{16,17}. Some effects were shown to be induced by an activation of enzymes *e. g.* monogalactolipase¹⁸, transacetylase¹⁹ or lipase²⁰, others might be due to chemical influences.

In this work the release of lipids from thylakoids in broken chloroplasts has been examined. Photochemical activities measured in parallel experiments demonstrated a strong correlation between the release of lipids and the decrease of photophosphorylation. Moreover, from the successive extraction of lipids from the membranes, the conclusions about the location of the different lipids in the membrane itself can be drawn.

Material and Methods

Chloroplasts from spinach were isolated as described by JACOBI²¹. The plant material was grown under defined conditions²². For the lipid analysis the chloroplasts were further purified by density gradient centrifugation according to LEECH²³.

The extraction and the fragmentation procedures are summarized in Fig. 1. After the chloroplasts had been suspended in salt media or in hypotonic solutions, the chloroplast extracts were separated by centrifugation at 10 000 x g. The composition of the media used is stated in the text and in the legends of

the Figs. The amount of lipids released from the membranes is determined from the analysis of the extracts.

The fragmentation of the thylakoids was carried out with a SCHÖLLER-sonifier as described^{24,25}. After 15 sec of sonication, the stacks were removed by centrifugation at 10 000 x g.

Lipids were extracted according to BLIGH and DYER²⁶. The lipids were transferred into the chloroform phase. After separation from the proteins, the evaporated extracts were again solubilized and taken for thin layer chromatography according to POHL *et al.*²⁷. Commercial plates (Woelm, Eschwege) were used throughout. The bands were extracted and analysed. The following methods were used for quantitative determinations: acyl ester²⁸, P_i ²⁹, galactose and glycerol enzymatically^{30,31}.

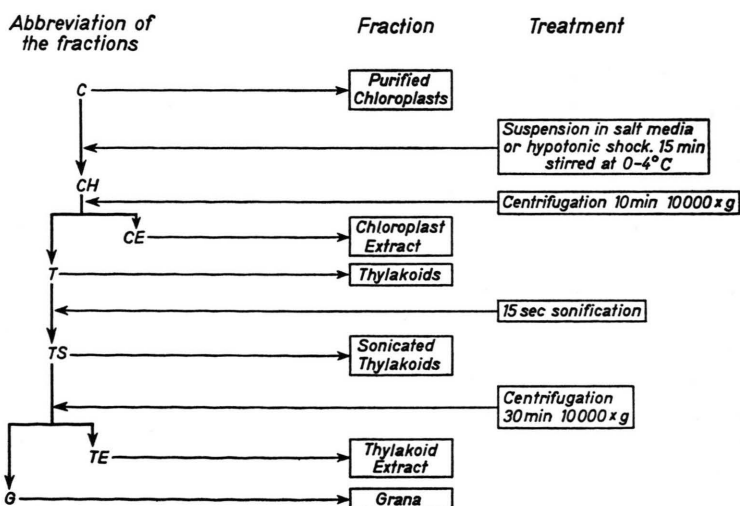
The sum of the compounds from the different fractions should be exactly identical to those values determined from the crude extract. Only when these two sets of values were equivalent, they were taken as representative. This fact is important especially for the data of P_i . The enormously high values in the crude extract cannot be used for the calculation of the total phospholipids. Significant amounts of P_i were found to be bound in the pigments.

Free and bound fatty acids were analysed by gas chromatography in the Packard-model 7300/7400. The column with a length of 3 m and a diameter of 4 mm was filled with 15% DEGS on gaschrom P.

Chlorophyll was determined according to ARNON³².

The light-induced reduction of ferricyanide and the formation of ATP was carried out in small Erlenmeyer flasks. The vessels contained (μ moles): Tricine buffer of pH 8.0 100; $MgCl_2$ 10; ADP 3; P_i 10; ferricyanide 6 and chloroplasts 0.1 mg chlorophyll/mg in a total volume of 3.0 ml. The basal rate was calculated from vessels which contained no ADP and P_i . The vessels were illuminated in Warburg appa-

Fig. 1. Preparation procedure.



ratus for 3 min at $6.1 \cdot 10^6$ ergs/cm² · sec at 20 °C. After adding 0.1 ml 70% HClO₄ and neutralization with KOH, the clear solution obtained after centrifugation was used for determinations. Ferricyanide was estimated by the difference of extinction at 420 nm between the light and the dark values. The ATP formed was determined enzymatically via hexokinase und glucose-6-phosphate dehydrogenase. All data presented were taken from triplicates.

The biochemicals were purchased from Boehringer & Soehne, Mannheim. The buffers came from Serva, Heidelberg. All other chemicals were of analytical grade from Merck, Darmstadt.

Results

The release of lipids from the thylakoid membranes and their stabilization

In the isolated chloroplasts purified by density gradient centrifugation, about 80% of the acyl lipids are galactolipids and minor amounts are phospholipids. The values summarized in Table I correspond to the

Table I. Lipid composition and lipid/chlorophyll ratios of purified chloroplasts.

	$\mu\text{mole Lipid}/$ $\mu\text{mole Chlorophyll}$	% of Total Lipid
MGD	1.24	33.0
MGD-X	0.51	13.5
DGD	0.76	20.2
DGD-X	0.37	9.8
SL	0.30	8.0
PG	0.28	7.4
PC	0.22	5.8
PI	0.06	1.8
PE	0.02	0.5

data reported in the literature for the lipid composition of chloroplasts¹⁻³. The majority of these compounds are constituents of the thylakoid membrane, but some of these lipids must also be assumed to be part of the outer envelope.

For the direct measurement of electron transport and the formation of ATP the use of envelope-free chloroplasts is required, due to the impermeability of the outer membrane for ADP and for the electron acceptors, such as ferricyanide or NADP⁺^{33,34}. Therefore, broken chloroplasts are used in the experiments concerning the investigation of electron transport and photophosphorylation. However, under these circumstances secondary effects might occur when the free thylakoidmembranes are suspended directly in an artificial medium.

Whenever the chloroplasts were broken in salt media^{35,36} or under hypotonic conditions, lipids were always found to be present in the supernatant after centrifugation at 10 000 x g. The lipids may derive from the thylakoids and also from the envelope. However the amount of lipids varies with the pH of the medium. Independent on the pH used, all chloroplasts are broken and the fragmented envelope is in the supernatant. Since the contribution of the lipids from the envelope should be constant under different conditions, the higher values should consequently account for the release from the thylakoids.

Fig. 2 summarizes the results obtained from an experiment in which the chloroplasts were suspended in salt media of different pH. From the data presented, it becomes obvious that the release of lipids is enhanced under alkaline conditions. The relative amount of phospholipids is significantly higher when compared to the galactolipids. However, when the lipids are analysed in detail, the different behaviour of MGD and DGD is obvious. In addition it can be seen from Fig. 2 that MGD became more easily liberated than DGD. The tendency of release is the same for all phospholipids and for SL. The highest values were always obtained for (PC+PI), followed by PG and SL. However, even at acidic pH, (PC+PI) showed the highest values, so that some of these compounds may derive from the envelope.

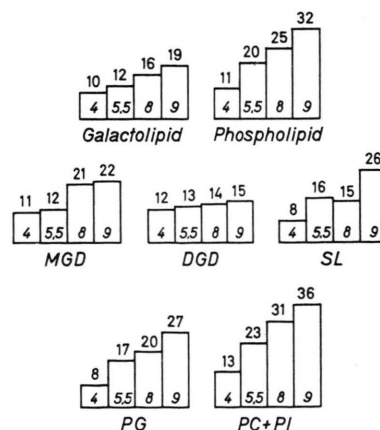


Fig. 2. Relative amount of lipids in extracts of salt-treated chloroplasts (CE) as a function of pH. The pH values of the medium is listed in the columns. In addition to 2% NaCl and 10⁻³M MgCl₂ the following buffers were used in a concentration of 10⁻²M: Acetate (pH 4.0); MES (pH 5.5); Tricine (pH 8.0); glycylglycine (pH 9). The concentration of the lipids in CE was compared with that of the thylakoids respectively (T+CE = 100%) in different experiments.

During the release of lipids secondary effects occur such as hydrolysis and the formation of derivatives. Under acid conditions, especially at pH 4, significantly higher amounts of free fatty acids were estimated by gas chromatography^(24a). Increasing amounts of derivatives of galactolipids have also been observed at pH 4 and 5.5. The derivatives were analysed to find out what proportion of galactose and glycerol they contained. The formation of acyl derivatives¹⁹ is unlikely because the expected amount of galactose was not found to be present. Therefore, it can be assumed that these compounds are degradation products. The values of galactolipids shown in Table I include the mono- and digalactolipids as well as their derivatives.

The experiment summarized in Fig. 2 was carried out in the presence of 2% NaCl. As already demonstrated²⁴, salts have a stabilizing effect on the configuration of the thylakoids. The stacks were shown to have a higher degree of packing in the contact zone of the grana area. The contact between the grana is loosened when the medium used for suspension contains low salt concentration.

Nevertheless, the tendency of release depending on the pH of the medium remained the same for all lipids when *no salts* were added. However, as shown in Fig. 3, the ratio MGD/DGD is the same in the chloroplast extract at pH 5.5, 8.0 and 8.9. This result is different from that found in salt media, where the relative amount of MGD is higher. From the comparison of the release under addition of NaCl and in salt-free media, the localization of DGD in the inner part of the membrane is assumed. Moreover, since measurable amounts of chlorophyll are found simultaneously with DGD in the supernatant at pH 8 and 8.9, part of this lipid is obviously in contact with the pigments. However, at pH 5.5 the supernatant does not contain any chlorophyll, but somewhat higher amounts of DGD are found compared with thylakoids treated with salt-media. Therefore, part of DGD might be also localized between the outer layer and the pigment area.

A reasonable explanation for the liberation of lipids from the membrane in alkaline media is that of an increasing dissociation of the polar groups, especially of the phospholipids. Consequently, stabilization should occur when the nucleophilic groups are neutralized by cations. On the other hand, when chelating agents like EDTA are added, a considerably greater release of lipids is expected.

As shown in Fig. 3 the amount of lipids present in the chloroplast extract is significantly higher when EDTA is present. The opposite effect is produced by the addition of $MgCl_2$. In order to get reproducible results, it was necessary to use a high concentration of Mg^{++} . However, the effects of Mg^{++} and of EDTA are both related to the pH. Increased liberation of lipids caused by EDTA as well as stabilization by Mg^{++} are more pronounced under alkaline conditions.

The influence of pH of the media on the photochemical activities of isolated chloroplasts

From the results obtained, the question arises as to whether the release of lipids is parallel to the change of photochemical activity. Chloroplasts that had been washed once were suspended in $10^{-3}M$ buffer of different pH without any other additions. The chlorophyll content of the suspensions was kept between 0.1–0.2 mg/ml. After standing for 15 min in the cold room, the broken chloroplasts were centrifuged at $25\,000 \times g$ and the pellet suspended in 0.1 M sucrose, 0.2% NaCl, 0.1% BSA and $10^{-4}M$ Tricine pH 7.5 (sucrose medium). As soon as the chlorophyll content

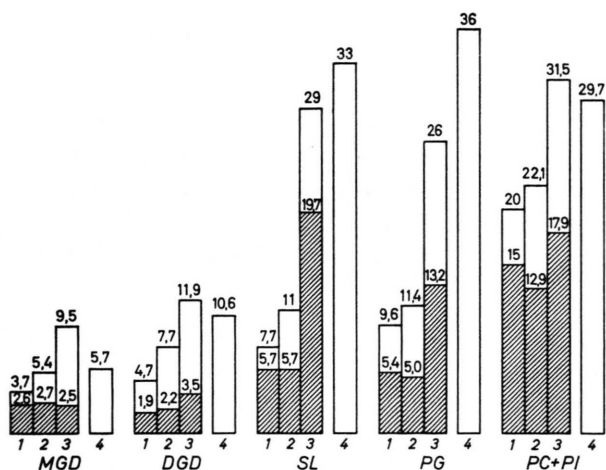


Fig. 3. Release of acyl lipids from a chloroplast suspension containing 1 μ mole chlorophyll after hypotonic shock at different pH. In this case the lipid compositions of the supernatants are compared with that of whole chloroplasts (s. p. 122), the latter is assumed as 100 %.

1. pH 5.5 ($10^{-3}M$ MES); MGD/DGD-ratio = 1.23 (2.05 with $10^{-2}M$ Mg^{2+}).
2. pH 8.0 ($10^{-3}M$ Tricine); MGD/DGD-ratio = 1.10 (1.92 with $10^{-2}M$ Mg^{2+}).
3. pH 8.9 ($10^{-3}M$ Glycylglycine). MGD/DGD-ratio = 1.24 (1.10 with $10^{-2}M$ Mg^{2+}).
4. pH 8.0 After addition of $10^{-3}M$ EDTA; MGD/DGD-ratio = 0.84.

The lower values shown in the columns represent the effect obtained after addition of Mg^{2+} ($10^{-2}M$).

had been determined and the suspensions adjusted to 1 mg chlorophyll/ml, the free thylakoids were measured for ferricyanide reduction and ATP formation.

As shown in Table II the phosphorylation is lost when the thylakoids are treated with media having a pH greater than 8. The uncoupling is indicated by

Table II. Photochemical activity of thylakoids after hypotonic shock at pH 5.5 and 8.9. Experimental conditions are given under „Material and Methods“.

	Electron transport*		ATP**	P/2e
	Basal	Coupled		
T 5.5	120	213	121	1.14
T 8.9	362	370	0	—

* μ Moles ferricyanide reduced/mg chlorophyll \times hour.

** μ Moles/mg chlorophyll \times hour.

the increased rate of electron transport. As expected, the proton gradient which has been measured simultaneously is lost. In contrast, a pH-shift could be demonstrated when the chloroplasts were suspended in media of pH 5.5; using a suspension with 68 μ g chlorophyll/ml, the pH rose from 5.26 to 5.37 after illumination at 10^6 ergs/cm² \cdot sec (SCHMIDT, SCHOPF, HEISE and JACOBI, in preparation). Furthermore, a stabilization of phosphorylation is found when the chloroplasts are broken at pH 5.5. Even after this treatment, the thylakoids isolated after centrifugation are very active in ATP formation. Values up to 250 μ moles of esterified P_i were found. Without subtraction of the basal rate³⁷, the P/2e ratio in the thylakoid fraction was always greater than one.

Nevertheless, the rate is lower as compared with freshly isolated chloroplasts suspended directly in the sucrose medium. Values between 500 and 600 μ moles of ATP formed/mg chlorophyll \times hour are normally observed in these „whole“ chloroplasts. The P/2e ratio is as high as 1.6–1.8^{38–40}.

The correlation between the release of lipids and the phosphorylation is further demonstrated by experiments using higher concentrations of MgCl₂ under alkaline conditions. As shown in Table III, the addition of 10⁻²M MgCl₂ significantly stabilizes the phosphorylation in the same way as found for the acyl lipids. When the thylakoids are suspended in 10⁻³M MgCl₂, the phosphorylation is not fully stabilized. However, in this range of concentration, there is a strong correlation between the concentration of salt in the suspension medium and the amount of chlorophyll released. Experiments with sodium chloride

Table III. Influence of Mg²⁺ upon the photochemical activity of thylakoids after hypotonic shock. Experimental conditions are given under „Material and Methods“ The values are given in μ moles/mg chlorophyll \times hour.

Medium	Buffer	Addition	Electron Transport		ATP	P/2e
			Basal	Coupled		
Tricin 8.5	—	—	459	518	0	—
Tricin 8.5	10 ⁻² M	MgCl ₂	143	311	176	1.13
MES 5.5	—	—	147	316	162	1.02
MES 5.5	10 ⁻² M	MgCl ₂	174	299	158	1.05
Tricin 8.5	—	—	460	515	14	0.05
Tricin 8.5	10 ⁻³ M	MgCl ₂	228	349	109	0.63

showed similar results, but Mg⁺⁺ was considerably more effective.

The release of lipids from extracted thylakoids by sonication

The different lipid composition of the supernatants obtained from thylakoids suspended in media of high and low ionic strength led us to conclude that some of these compounds may be located in the outer part of the membrane. Compared with the composition of intact chloroplasts, pronounced differences were found especially for the higher ratio of MGD/DGD in extracts of salt treated chloroplasts. DGD has already been shown to be liberated more easily by a hypotonic shock (Fig. 3). Therefore, when the membranes are treated more drastically, one would expect DGD to be released in higher quantities. So greater effects should result from sonication experiments.

For the experiments with sonication the extracted thylakoids were again suspended in a medium of high salinity which had previously been shown to stabilize the contact of the grana^{24,41}. After 15 sec of sonication, the grana stacks were sedimented by centrifugation. The supernatant and the sediment were analyzed for the lipid composition.

In the same way as shown in Fig. 2, higher amounts of lipids were also liberated under alkaline conditions after sonication. However, as seen from Fig. 4, the composition of the supernatant (TE) differs from that of the chloroplast extract. It contains especially higher amounts of chlorophyll and a lower ratio of MDG/DGD.

Most of the chlorophyll in the supernatant comes mainly from the intergrana area. This conclusion is drawn from electronmicrographs which were published

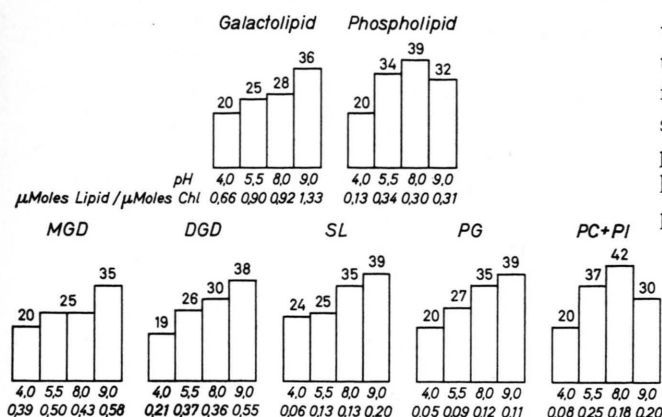


Fig. 4. The release of lipids from extracted thylakoids after sonication. The numbers at the top of the columns express the percentage of release in relation to the thylakoid values. The pH used and the absolute values in $\mu\text{moles lipid}/\mu\text{moles Chl}$ are listed under the columns ($G+TE = 100\%$).

previously^{24,41}. In these studies the fractions obtained after sonication were characterized. The functional state of the grana stacks isolated from sonicated chloroplasts has also been described^{24,25}.

The analysis of fatty acids — not shown here — demonstrated secondary chemical transformations as indicated by the rise of $C_{12:0}$ fatty acids. The amount of free fatty acids inside the grana is significantly higher at acid pH^{24a}.

The relative lower value of phospholipids at pH 9, especially of the fraction (PC+PI) is due to the fact that the free thylakoids used for the sonication contained less lipids. The fraction represents free thylakoid systems which were preextracted at the same pH and thereafter separated by centrifugation. Since higher amounts of polar lipids were extracted already in the first step, less quantities are expected to be present in the supernatant after sonication.

The binding of released lipids with particles

Under alkaline conditions lipids were seen to be liberated at the same time as the phosphorylating activity decreased or even disappeared. The coincidence prompted the idea that factors involved in coupling are removed. The localization of the coupling factor CF_1 at the surface has been demonstrated. Moreover, it has been shown that this protein is released by EDTA¹²⁻¹⁴ and stabilized by Mg^{++14} .

Chloroplasts were suspended in $10^{-2}M$ glycylglycine pH 8.9 containing $5 \cdot 10^{-4}M$ EDTA. In order to get higher values of lipids in the supernatant, a treatment

with the YEDA-press was included. After centrifugation for 30 min at $10\,000 \times g$, the supernatant was further centrifuged at $170\,000 \times g$ for 30 min. As shown in Fig. 5 most of the lipids were found in the pellet. This result indicates that the majority of the lipids released from the membranes are bound to particles.

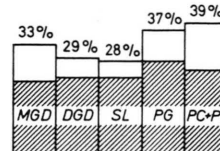


Fig. 5. The amount of sedimentable lipids (hatched part of the columns) from the supernatant of chloroplast extracts obtained by "Yeda-Press" treatment.

The analysis of the supernatant after the removal of the particles showed a rise of MGD and consequently, higher values of the ratio MGD/DGD are calculated. Therefore, MGD becomes solubilized when extracted from the membranes. In contrast, most of the phospho- and sulfolipids are located in the pellet.

Discussion

The aim of the investigations that are presented in this paper was to gain information about the localization of the different lipids in the thylakoid membranes. Furthermore, it was hoped to find a correlation between a change in photochemical activities and the lipids released from the membranes.

In order to get suitable information, it was necessary to find conditions under which the degradation occurs in stages. An invaluable and simple method to release the lipids from the surface of the thylakoids was found by the treatment with media of higher pH. Whenever the chloroplasts were broken between pH 8 and 9, appreciable amounts of acyl lipids were liberated. This phenomenon is observed only when the chlorophyll content of the suspension is approximately 0.1 mg/ml or even lower. However, dependent upon the pretreatment and thus on the state of the whole thylakoid system, the release of lipids was found to be different. Using salt media, the ratio MGD/DGD was greater in the extract. In contrast, the liberation of DGD and of the pigments increased considerably under hypotonic conditions and even more by EDTA and by sonication. Consequently, DGD and the pig-

ments are suggested to be localized in the inner part of the membranes.

The results contradict with those of KREUTZ⁶ but differ also from the assumption made by WINTERMANS⁴². WINTERMANS' conclusion came from sonication experiments. The identity of the MGD/DGD ratio in the particles of different size have led him to propose that both types of lipids are distributed uniformly in the membranes. However, as demonstrated in our experiments, the relatively greater release of MGD is only observed when the thylakoids are suspended in salt media. Using hypotonic solutions or after treatment with sonication our data are equivalent with those published by WINTERMANS. From the results obtained in both laboratories the conclusion can be drawn that the lipid composition of the grana- and the stromamembranes in mature chloroplasts are identical. Therefore, it still remains an open problem, which compound is responsible for the stacking.

Our results are explained best when the concept of hydrophobic bonds between lipids and proteins as first proposed by WEIER and BENSON⁹ and more recently by BENSON *et al.*¹⁰ is taken in consideration. These investigators suggest that there is a hydrophobic interior of protein globules in which the acyl groups of the lipids are buried. Thus, the exterior of the protein globules which are in contact with the other membrane constituents, should be surrounded by polar dissociable groups, especially those of esterified phosphorus- and sulfonic acids. On the basis of this concept, the polarity increases by lowering the proton concentration. As a consequence, the lipids become more hydrophilic and together with the proteins they would be discharged from the anionic membrane surface.

This interpretation coincide fairly well with the conclusions drawn from experiments concerning the protonation of the membrane⁴³. The protonation of negatively charged groups, resulting in membrane conformation⁴⁴, has already been proposed as a primary event of photosynthesis⁴⁵. Furthermore, the chloroplasts were shown to undergo reversible volume changes during illumination or even in the dark by the addition of acids or alkali⁴⁶. MURAKAMI and PACKER⁴⁷ extended these studies by investigating the configuration changes with the use of electron microscopy. The thylakoids were found to shrink during illumination or by lowering the pH in the dark. The thickness of the single membranes was found to be changed only by protons or by cations, but not by

sucrose. The stabilization of lipids by cations at higher pH can be explained in the same way. As demonstrated further by GROSS and PACKER⁴⁸, the uptake of cations dependent on light results in a contraction of membranes.

Only the acid groups from the phospho- and sulfolipids can be responsible for the fact that the polar groups change their dissociation. This change is dependent upon the proton concentration which decreases in light. The tendency of release observed for MGD is still unclear. MGD was found to be synthesized at the very early stage during the development of the thylakoid system⁴⁹⁻⁵³. It might be possible that MGD participates in an orientation at the surface and is less bound to proteins (Fig. 5). The predominantly unsaturated acyl residues of this lipid⁵⁴ might also favour the permeability for non-polar molecules⁵⁵.

However, the data presented in this paper are not conclusive in respect to the nature of the proteins which are released simultaneously with the lipids. The proteins liberated under alkaline conditions are possibly different from the coupling factor CF_1 ¹¹. Several experiments demonstrated the release of the ATP-ase by EDTA. As shown in Fig. 3 the amount of lipids in the chloroplast extract is considerably enhanced by EDTA. Most recently, experiments in our laboratory demonstrated that the ATP-ase remained at the membrane when the chloroplasts are suspended at pH 8.9. (SCHMID, SCHOPF, HEISE, and JACOBI, in preparation). However, a regeneration of phosphorylation and of the proton gradient is achieved when the supernatant and the extracted thylakoids are mixed with high concentrations of Mg^{++} . Consequently, the lipids or the lipoproteins in the alkaline supernatant represent another „coupling factor“ which is required for the phosphorylation.

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