Antibodies against Sulphoquinovosyl-diacyl glycerol and their Reactions with Chloroplasts

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Using immunological techniques we tried to determine the localization and orientation of the sulpholipid of chloroplasts in thylakoids. During immunization of rabbits with lamellar systems of chloroplasts of *Antirrhinum majus* antisera were obtained which precipitated sulpholipid emulsions. Antibodies against the sulpholipid were not detected until a second series of intravenous injections.

By immunizing purified sulpholipid of *Fucus serratus*, adsorbed to methylated bovine serum albumin, sulpholipid antisera were acquired. These antisera and the antisera against lamellar systems of *Antirrhinum majus* reacted with preparations of sulpholipid from widely different plant species. The sulpholipid antisera did not react with emulsions of the mono- or digalactosyl-diacyl glycerol, of lecithin or the anionic phosphatide phosphatidyl glycerol. Lamellar systems isolated without osmotic shock on a sucrose gradient were not agglutinated by sulpholipid antisera directly, but an indirect agglutination could be achieved in a Coombs-test or in a mixed antigen agglutination after Uhlenbruck. Broken chloroplasts, however, were agglutinated directly by sulpholipid antisera, as well lamellar systems which had been disrupted in a French-press or by ultrasonic treatment.

From agglutination and agglutination inhibition experiments with lamellar systems prepared by different methods it is concluded that the determinant groups of the sulpholipid are accessible for antibodies in the lamellar system of chloroplasts. But probably the sulpholipid molecules are located inside the thylakoids.

Abbreviations: Sulpholipid = Sulphoquinovosyl-diacyl glycerol = 6-sulpho-6 deoxy- α -D-glucopyranosyl diglyceride, Tris buffer = Tri(hydroxymethyl)-aminomethane HCl buffer, EDTA = Ethylenediamine tetra-acetic acid, NADP⁺ = Nicotinamide adenine dinucleotide phosphate, oxidized form, BSA = bovine serum albumin, saline = 0.85% aqueous solution of NaCl.

Earlier investigations with antisera against preparations of ferredoxin-NADP⁺ reductase¹⁻³ and preparations of solubilized lamellar structural protein of chloroplasts^{4,5} have shown that it is possible to localize these components at that surface of the lamellar system which is accessible for antibodies. By immunizing rabbits with isolated lamellar systems antisera were obtained which agglutinated emulsions of ether soluble lipids of chloroplasts. Using these antisera it has been attempted to localize the lipids in the thylakoid membrane⁴.

In this publication it is shown that these antisera contained antibodies against the sulpholipid of chloroplasts. Therefore a localization of the sulpho-

lipid and an investigation about the orientation of this lipid in the thylakoid membran appears possible with antigen-antibody reactions. For this purpose it was very useful to get antibodies against the sulpholipid by immunizing with the sulpholipid itself.

Methods and Experiments

1. Isolation of lipids

To obtain the sulphoquinovosyl-diacyl glycerol from plant material the ether soluble lipids were separated chromatographically on columns of SiO₂ (Mallinckrodt, 100 mesh, Roth, Karlsruhe, Germany), Diethyl aminoethyl cellulose MN 2100 DEAE, Macherey, Nagel & Co, Düren, Germany) and florisil (60–100 mesh, Roth, Karlsruhe, Germany). The preparation, identification and fatty acid composition of the different sulpholipids have already been described⁶. After two dimensional thin-layer chromatography of the isolated sulpholipid fractions on Silicagel G plates in different solvents

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** Preliminary experiments for this publication were carried out in the Institut of Botany at the University of Köln, Germany.

¹ R. BERZBORN, W. MENKE, A. TREBST, and E. PISTORIUS, Z. Naturforsch. **12b**, 1057 [1966].

² R. BERZBORN, Z. Naturforsch. **23b**, 1096 [1968].

³ R. BERZBORN, Z. Naturforsch. **24b**, 436 [1969].

⁴ R. BERZBORN, Dissertation, Köln 1967.

⁵ R. BERZBORN and W. MENKE, Z. Naturforsch., in preparation.

and after spraying with different reagents, only one spot appeared⁶.

Mono- and digalactosyl-diacyl glycerol were also separated out of the ether soluble lipids of *Fucus serratus* and *Antirrhinum majus* by column chromatography on the same adsorbents. The galactose of both lipids was identified by thin-layer- and gas chromatography⁷.

Phosphatidyl glycerol was isolated out of the ether soluble lipids of *Rhodospseudomonas spheroides* on DEAE cellulose columns and was purified on SiO₂ columns after ROTSCH and DEBUCH⁸. Lecithin was prepared from the commercially available egg lecithin (Merck, Darmstadt, Germany). Further purification was obtained with DEAE cellulose and SiO₂ column chromatography.

2. Immunization with lamellar systems

Rabbits were immunized by a series of intravenous injections with lamellar systems isolated out of *Antirrhinum* chloroplasts in sucrose solutions^{4, 5}. Five months after completion of the first series the animals received a second series of intravenous injections, five months after completion of this series they got a third series, some animals even got a fourth one. Instead of the first series some animals were immunized with a mixture of lamellar systems and Freund's Adjuvant (compl., Difco No. 0638-60, Difco Lab., Detroit, Michigan, USA) and boosted after 6 weeks⁴. These animals later received series of intravenous injections according to the same schedule than the other rabbits (Fig. 1).

3. Immunization with sulpholipid adsorbed to methylated BSA

Commercially available BSA (fraction V, Calbiochem, Los Angeles, California, USA) was dissolved in methanol and methylated with HCl⁹. The methylated albumin was spun down and washed twice with methanol, twice with ether, and then dried in vacuo over P₂O₅. According to the method of INOUE and NOJIMA¹⁰, * 30 mg sulpholipid and 30 mg lecithin, in a second experiment 3 mg sulpholipid and 30 mg lecithin, and in a third experiment only 30 mg sulpholipid were dissolved in 5 ml ethanol, mixed with 5 ml saline (0.85% NaCl in H₂O) and thoroughly shaken by hand in a 50 ml round bottom flask for 2 to 5 min. All the ethanol was evaporated in vacuo to about one third of the volume, distilled water was added to a final volume of 5 ml and 5 ml of an aqueous solution of 0.1% (W/V) methylated BSA was added and again thoroughly

shaken. The suspensions were prepared 12 h before the injections and kept at 4°. In each experiment 5 rabbits were given 14 intravenous injections of 2 ml of the preparations every other day (Table 1). Lecithin was adsorbed to methylated BSA after the same method as sulpholipid, methylated BSA was injected in saline. In both cases two rabbits were immunized with these antigen for control experiments (Table 1).

4. Methods to demonstrate antibodies against sulpholipid

a) Agglutination on slides (slide test): Isolated lamellar systems were suspended in saline, 0.066 M phosphate buffer (KH₂PO₄ + Na₂HPO₄) (pH 7.4) or 0.02 M Tris buffer (pH 7.4) to give a final concentration of 1.5 mg chloroplasts (dry weight)/ml. Sulpholipid and the other lipid fractions were suspended also in saline, or the same buffer, to give a final concentration of 3 mg lipid/ml aqueous solution. The suspension was then emulsified by ultrasonic treatment 2 times for 30 sec. in an icebath (MCE Ultrasonic Power Unit, 2.0 Amp.). Because the lipids aggregated a modified latex tests after SINGER and PLOTZ¹¹ and after WIEDERMANN¹² were also carried out. Assuming the density of the lipids to be 1 the amount of lipid was calculated⁴, to give a bimolecular lipid layer on the surface of the latex particles. 50 µg lipid were emulsified in 1 ml saline with a suspension of 0.2% (V/V) latex particles with a diameter of 0.79 µ (Polystyrene Latex, The Dow Chemical Comp., Midland, USA). Equal volumes of these preparations and of the sera under investigation were mixed on the slide, the slide gently moved for some seconds and the reaction observed in the microscope.

b) Agglutination in tubes (micro tube test): 0.04 ml of the antigen suspension and 0.04 ml of a serum in question were mixed in small round bottom glass tubes (4 mm diameter). The reaction mixture was incubated for 2 h at 20° and 16 h at 4°. During this time all particles settled down and the supernatants became clear. But the small pellets showed characteristic patterns. Similar to the patterns which developed, when agglutinating antibodies reacted with lamellar systems⁴, latex particles with the sulpholipid showed after a reaction with sulpholipid antibodies coarse pellets with notched folded rims or less coarse pellets with minute folded rims depending on the amount of antibodies in the mixture. Standard serum led to homogeneous pellets with smooth rims in adequate mixtures.

c) Determination of the titer: The amount of the antibodies in the antisera could only be determined

⁶ A. RADUNZ, Hoppe-Seyler's Z. physiol. Chem. **350**, 411 [1969].

⁷ A. RADUNZ, Hoppe-Seyler's Z. physiol. Chem. **349**, 303 [1968].

⁸ E. ROTSCHE and H. DEBUCH, Hoppe-Seyler's Z. physiol. Chem. **343**, 135 [1965].

⁹ N. SUCOKA and T. Y. CHENG, J. molecular Biol. **4**, 161 [1962].

¹⁰ K. INOUE and S. NOJIMA, Biochim. biophysica Acta [Amsterdam] **144**, 409 [1967].

* Lipid were adsorbed on methylated BSA. In the first experiment.

¹¹ J. M. SINGER and C. M. PLOTZ, Americ. J. Med. **21**, 888 [1956].

¹² G. WIEDERMANN, Zbl. Bakt., Abt. I Orig., Sonderheft **278** [1963].

semiquantitatively by analyzing the titer of the agglutination. To determine agglutinating antibodies against lamellar systems 0.04 ml saline were put in 15 small test tubes (4 mm diameter) 0.04 ml serum added to the first one, mixed and 0.04 ml of the diluted serum transferred to the next tube etc. to make geometric progression with a dilution factor of 0.5 (or 1 : 2). To each tube of this dilution series 0.04 ml of a suspension with lamellar systems (1.5 mg/ml in saline) was added. After 2 h at 20° and 16 h at 4° the reaction was judged after the patterns of the pellets⁴.

To determine the antibodies against sulpholipid 0.12 ml serum was added to the first tube with 0.04 ml saline, mixed and 0.12 ml of the diluted serum transferred to the second tube with 0.04 ml saline etc. to make a geometric progression with a dilution factor of 0.75 (or 3 : 4). 0.04 ml of a sulpholipid emulsion (1 mg/ml in saline) was added to each tube and mixed. After 2 h at 20° the reaction was judged by observing droplets of the mixture in the microscope. To be sure whether a reaction was positive or negative, equal dilution series were made in every case with standard serum.

5. Experiments to agglutinate lamellar systems indirectly by sulpholipid antisera

a) Mixed antigen agglutination after UHLENBRUCK¹³: 0.04 ml of a suspension of lamellar systems (1.5 mg/ml in saline), which were isolated in sucrose solutions was mixed on slides with 0.04 ml sulpholipid antiserum and gently agitated for 30 sec or more. Then 0.04 ml of a sulpholipid emulsion after ultrasonic treatment (3 mg/ml in saline) was added, and the slides with the droplet gently moved again. The first and the second step of the reaction was examined in the microscope (Table 3).

b) Coombs-Test¹⁴: The first step of this reaction is the same as described above. In the second step 0.04 ml of an antiserum against rabbit- γ -globulin was added (Difco No. 2366-50, Difco Lab Detroit, Michigan, USA). In both cases control experiments were carried out with standard sera, antisera against methylated BSA and saline (Table 3).

c) Washing experiment: To remove all antibodies not bound to the surface of the thylakoids in the first step of the mixed antigen agglutination, 0.2 ml of sulpholipid antiserum and 0.2 ml of a suspension of lamellar systems (1.5 mg/ml in saline) were mixed in centrifuge tubes. After 2 h at 20°, and in a second experiment after an additional 16 h at 4°, the lamellar systems were spun down for 10 min at 2000 g and 4°. The pellet was washed twice in 5 ml 0.85% NaCl solution and spun down. Finally the incubated lamellar systems were suspended in 0.2 ml 0.066 M phosphate

buffer (pH 7.4) and droplets of this preparation was mixed on slides with the sulpholipid emulsion or with the antiserum against rabbit- γ -globulin, as described above.

Results

1. Antibodies against sulpholipid in antisera against lamellar systems of chloroplasts

When rabbits were immunized with isolated lamellar systems of chloroplasts of *Antirrhinum majus* by one or more series of intravenous injections*, or by help of Freund's Adjuvant**, antisera were obtained which reacted with several different preparations of soluble and insoluble components of chloroplasts^{1, 4, 5}. Some of the antisera even agglutinated emulsions of ether soluble lipids of chloroplasts.

To clarify the question to which lipid of the chloroplasts the rabbits make antibodies against, when injected with lamellar systems, monogalactosyl-diacyl glycerol, digalactosyl-diacyl glycerol, sulphoquinovosyl-diacyl glycerol and phosphatidyl glycerol out of the chloroplasts were separated chromatographically on columns of SiO₂ diethylamino ethyl cellulose and florisisil^{6, 7}. Only the chromatographically pure sulpholipid was agglutinated by the antisera against lamellar systems.

During and after the series of injections the appearance of antibodies against lamellar systems and sulpholipid in the blood serum was observed by estimating the titer of agglutination semiquantitatively. Two rabbits synthesized sulpholipid antibodies during the second series of intravenous injections and two other rabbits during the third series. No difference was found in the appearance of sulpholipid antibodies when the rabbits got an injection of a mixture of lamellar systems with Freund's Adjuvant and two booster injections after 6 weeks instead of a first series of intravenous injections during 2 months.

Fig. 1 shows the titer of the sera of one rabbit. After the second series of injections the antisera agglutinated a sulpholipid emulsion (0.1% in saline) down to a dilution of 1 : 3. The titer of

¹³ O. PROKOP and G. UHLENBRUCK, in: Lehrbuch der menschl. Blut- und Serumgruppen II, Edition, VEB Thieme, Leipzig 1966; G. UHLENBRUCK and O. PROKOP, Dtsch. med. Wschr. **92**, 940 [1967]; G. UHLENBRUCK, Med. Welt **17**, 906 [1965].

¹⁴ R. R. A. COOMBS, M. H. GLEESON-WHITE, and J. G. HALL, Brit. J. exp. Pathol. **32**, 195 [1951].

* We thank Dr. G. UHLENBRUCK for his advice for this immunization schedule.

** We thank Dr. K. RAJEWSKY for his advice for this immunization schedule.

Fig. 1. Titer of agglutinating antibodies in the serum of one rabbit during four series of immunization with isolated lamellar systems of chloroplasts.

antibodies against sulpholipid could be increased by a third and a fourth series of intravenous injections of lamellar systems. At the end of the third series the sera gave a clear positive reaction down to a dilution of 1 : 10 and after the fourth series down to a dilution of 1 : 14.

2. Antisera against sulpholipid and their characterization

For the purpose of immunization the sulpholipid was purified from the ether soluble lipids of *Fucus serratus*⁶. Rabbits were injected every other day with sulpholipid adsorbed to methylated bovine serum albumin (BSA) after INOUE and NOJIMA¹⁰. The precise immunization schedule is given under experiments and in Table 1. After 12 injections the blood serum of each animal contained antibodies against sulpholipid. INOUE and NOJIMA found that antibodies against cardiolipin are only synthesized when the cardiolipin was adsorbed to methylated BSA together with lecithin. To obtain antibodies against sulpholipid this was not necessary. Antisera obtained by injections of sulpholipid adsorbed to

methylated BSA reacted with sulpholipid emulsion (0.1% in saline) down to a dilution of 1 : 10 as well as antisera which were obtained by injection of sulpholipid absorbed together with lecithin to methylated BSA. The amount of antibodies in the sera could not be increased by further injections. The titer of the antisera, however, was dependent on the amount of sulpholipid injected to each animal. When the rabbits were injected with 0.6 mg instead of 6 mg sulpholipid every other day, the antisera reacted only until a dilution of 1 : 3.

The sulpholipid antisera obtained by immunization with the sulpholipid of the brown alga *Fucus serratus* also reacted to the same extent with the sulpholipids isolated by the same procedure out of the higher plant *Antirrhinum majus*, the fern *Dryopteris filix-mas*, the red alga *Batrachospermum moniliforme*, the blue-green alga *Oscillatoria chalybea* and the purple bacterium *Rhodospseudomonas spheroides*, although there were remarkable differences in the quality and quantity of the fatty acids of the sulpholipids of these organisms⁶. Hydrogenized sulpholipid emulsions reacted with the antisera, but

Composition and amount of the antigens for each injection	number and kind of injection	number of animals with antibodies against sulpholipid/number of treated animals	titer against sulpholipid
1. 6 mg sulpholipid and 6 mg lecithin in 2 ml 0.05% solution of methylated BSA	14 in the ear vein	5 / 5	1 : 10
2. 0.6 mg sulpholipid and 6 mg lecithin in 2 ml 0.05% solution of methylated BSA	14 in the ear vein	5 / 5	1 : 3
3. 6 mg sulpholipid in 2 ml 0.05% solution of methylated BSA	14 in the ear vein	2 / 2	1 : 10
4. 6 mg lecithin in 2 ml 0.05% solution of methylated BSA	14 in the ear vein	0 / 2	—
5. 1 mg methylated BSA in 2 ml saline	14 in the ear vein	0 / 2	—

Table 1. Immunization schedule to obtain antisera against sulpholipid, lecithin and methylated bovine serum albumin.

after sodium periodate treatment¹⁵ for 16 h no reaction of the lipid with the antisera could be obtained. Therefore in this case the sulphoquinovose portion seems to be the reacting antigen determinant.

To examine the specificity of the sulpholipid antisera the mono- and digalactosyl-diacyl glycerol, lecithin and the anionic phosphatide phosphatidyl glycerol, also found in chloroplasts¹⁶, were tested. None of these lipids reacted with antisera against sulpholipid.

3. Reaction of sulpholipid antisera with different chloroplast preparations

For the immunological experiments the lamellar systems from *Antirrhinum* chloroplasts were isolated in sucrose solutions, thereby the soluble proteins were removed¹⁷. Suspensions of the lamellar systems were tested on slides or in small tubes. Sulpholipid antisera did not agglutinate with lamellar systems directly. This was expected since it is not possible to decrease the extend of agglutination of lamellar systems by antisera against lamellar systems by preincubation of the antisera with a mixture of acetone soluble lipids⁴ or with emulsions of purified sulpholipid at increasing concentrations (Table 2, C).

It is known that concentrated solutions for albumin have a hydrating effect on the outer layer of the membrane of the red blood cells¹⁸. This allows some antibodies to agglutinate the red blood cells

in this solution, but not in saline. Therefore the lamellar systems were suspended in a solution of 20% albumin and tested with sulpholipid antisera, but no agglutination was noted.

A possible interpretation of this finding might be that the sulpholipid is solated inside the thylakoid membrane and the determinant group would not be accessible for the antibodies. Another possible explanation might be that a reaction of the antibodies with the sulpholipid takes place, but no agglutination of the particles is to be seen, because the reacting determinant of the sulpholipid is located in depressions in the surface of the lamellar systems. After treatment with EDTA¹⁹ lamellar systems were also not agglutinated by sulpholipid antisera directly. An analogous experiment with ferredoxin-NADP⁺ reductase antisera^{3, 4} were positive, i.e. lamellar systems treated with EDTA were agglutinated directly.

Next, agglutination inhibition experiments were carried out. As seen in Table 2 an antiserum against lamellar systems, which reacted with a sulpholipid emulsion down to a dilution of 1 : 24, did not react with sulpholipid emulsions, when it was absorbed with a suspension of 15 mg lamellar systems/ml or more, when equal volumes of the undiluted serum and the antigen suspension were mixed. Sulpholipid antiserum, which reacted with a sulpholipid emulsion down to a dilution of 1 : 10, was completely absorbed by adding equal volumes of a suspension

¹⁵ E. A. KABAT and M. M. MAYER, in: *Experimental Immunochimistry*, C. C. Thomas Ed., Springfield, Illinois, USA, 1961, p. 542.

¹⁶ A. T. JAMES and B. W. NICHOLS, *Nature [London]* **210**, 372 [1966]; F. HAVERKATE and L. L. VAN DEENEN, *Biochim. biophysica Acta [Amsterdam]* **106**, 78 [1965].

¹⁷ H. NICKEL, *Flora Abt. A* **159**, 233 [1968].

¹⁸ H. J. PETTENKOFER, in: *Blutgruppenserologie*, Beiheft zu den *Laboratoriums-Blättern der Behring-Werke*, 1961, Heft 5.

¹⁹ A. T. JAGENDORF and M. SMITH, *Plant Physiol.* **37**, 135 [1962]; M. AVRON, *Biochim. biophysica Acta [Amsterdam]* **77**, 699 [1963].

concentration of the antigens for the absorption	a) absorption with lamellar systems		b) absorption with ultrasonically treated lamellar systems		c) absorption with sulpholipid	
	supernatant lamellar systems	reacts with sulpholipid	supernatant lamellar systems	reacts with sulpholipid	supernatant lamellar systems	reacts with sulpholipid
2.5 mg/ml	++++	++++	++++	++++	++++	++++
5.0 mg/ml	++++	++++	++++	++	++++	+++
10.0 mg/ml	+++	++	+++	±	++++	++
15.0 mg/ml	+++	±	++	—	++++	+
20.0 mg/ml	++	—	—	—	++++	—
30.0 mg/ml	+	—	—	—	++++	—
35.0 mg/ml	—	—	—	—	++++	—

Table 2. Agglutination inhibition experiments. 0.2 ml undiluted antiserum against lamellar systems with a titer against lamellar systems of 1 : 2048 and a titer against sulpholipid of 1 : 24 was mixed with 0.2 ml of suspensions with increasing concentrations of lamellar systems, ultrasonically treated lamellar systems and sulpholipid emulsions (2.5 to 35 mg/ml saline). After 2 h at 20° the reaction mixtures were centrifuged (5 min at 13000 g). The supernatants were tested with a suspension of lamellar systems (1.5 mg/ml in saline) and with sulpholipid emulsions (1 mg/ml in saline), using the slide test.

of 10 mg lamellar systems/ml or more. When the sera were absorbed with ultrasonically treated lamellar systems, 10 mg/ml was enough for the antiserum against lamellar systems and 5 mg/ml for the sulpholipid antiserum to absorb completely the antibodies against sulpholipid. Apparently, after ultrasonic treatment more determinants are exposed which then react with the sulpholipid antibodies.

These experiments showed that the determinant groups of the sulpholipid itself must be accessible for antibodies on the surface of the thylakoid stacks. Excluded is the unspecific reaction of sulpholipid antibodies with the surface of the lamellar systems²⁻⁴. But as written before the lamellar systems were not agglutinated by the sulpholipid antisera directly. The easiest way to explain this apparent contradiction is to suggest that the sulpholipid is located in depressions of the surface of the isolated particles as mentioned above. To test this hypothesis, experiments were carried out after the experimental design of mixed antigen agglutination after UHLENBRUCK¹³ and the Coombs-test¹⁴ (Table 3). Suspensions of lamellar systems were mixed on slides with a sulpholipid antiserum, and after 30 sec an emulsion of sulpholipid or an antiserum against rabbit- γ -globulin was added. In both cases lamellar systems were agglutinated indirectly in the second step of the reaction. From the control experiments listed above it is clear that the reaction of sulpholipid antibodies with the lamellar systems in the first step of the reaction is specific. Beside this, a positive reaction only could be achieved in the second step as an indirect agglutination, when the three components of the reaction were mixed in proper amounts in the right order (Table 3), and if the time for the

first step of the reaction was not too short. These phenomena are discussed in earlier publications²⁻⁴. Therefore lamellar systems were not unspecifically coprecipitated with the agglutinates of the sulpholipid emulsions and sulpholipid antibodies, and they were not unspecifically coprecipitated with the sulpholipid antibodies and other γ -globulins of the rabbit blood sera by the γ -globulin antisera added in the second step. But sulpholipid antibodies reacted with determinants of sulpholipid molecules in the surface of the isolated particles.

Washing experiments gave the same result. Lamellar systems isolated in sucrose solutions were incubated with sulpholipid antisera, and all antibodies not bound to the surface of the particles were washed off with saline. Nevertheless the incubated lamellar systems were agglutinated indirectly by addition of an emulsion of sulpholipid or an antiserum against rabbit- γ -globulin.

Because it is not known to what extent the lamellar systems isolated in sucrose solutions are still surrounded by the chloroplast envelope, this structure could be the reason for the described agglutination behaviour of the particles with sulpholipid antisera. Thus the result of the indirect agglutinations would only allow some conclusions about the location of the sulpholipid in the thylakoid, but not about the surface structure of the lamellar systems. Therefore broken chloroplasts from *Antirrhinum* leaves were isolated according to the procedure of ALLEN²⁰ et al., and tested. These particles were agglutinated directly by sulpholipid antisera in con-

²⁰ M. B. ALLEN, F. R. WHATLEY, and D. I. ARNON, *Biochim. biophysica Acta* [Amsterdam] **32**, 32 [1959].

component No. 1	component No. 2	first reaction	component No. 3	second reaction
1. lamellar systems	sulpholipid antiserum	±	saline	±
2. lamellar systems	sulpholipid antiserum	±	sulpholipid	green agglutinates
3. sulpholipid antiserum	sulpholipid	white agglutinates	lamellar systems	lamellar systems remain homogeneously distributed green and white agglutinates
4. lamellar systems	sulpholipid	—	sulpholipid antiserum	
5. lamellar systems	sulpholipid antiserum	±	standard serum	±
6. lamellar systems	sulpholipid antiserum	±	rabbit-γ-globulin antiserum	green agglutinates
7. sulpholipid antiserum	rabbit-γ-globulin antiserum	white precipitates	lamellar systems	lamellar systems remain homogeneously distributed only white precipitates
8. lamellar systems	rabbit-γ-globulin antiserum	—	sulpholipid antiserum	
9. lamellar systems	sulpholipid antiserum	±	methylated BSA	±
10. lamellar systems	methylated BSA antiserum	—	methylated BSA	only white precipitates
11. lamellar systems	methylated BSA antiserum	—	sulpholipid	—
12. lamellar systems	methylated BSA antiserum	—	rabbit-γ-globulin antiserum	only white precipitates
13. lamellar systems after ultrasonic treatment	sulpholipid antiserum	green agglutinates		
14. broken chloroplasts	sulpholipid antiserum	green agglutinates		
15. lamellar systems	lamellar systems antiserum	green agglutinates		

Table 3. Indirect and direct agglutination experiments and controls (slide tests). Lamellar systems were isolated in sucrose solutions, broken chloroplasts in NaCl solutions and suspended, and the pure sulpholipid suspended and emulsified as described under experiments and methods. Undiluted sera were used, and the concentration of antigens were: lamellar systems 1.5 mg/ml; lamellar systems after ultrasonic treatment 1 mg/ml; broken chloroplasts 1.5 mg/ml; sulpholipid 3 mg/ml, and methylated BSA 1 mg/ml; all in saline.

trast to the antisera against ferredoxin-NADP⁺ reductase, which agglutinated those particles only indirectly. According to electron microscope studies²¹ broken chloroplasts consist of thylakoid stacks which are not surrounded by the chloroplast envelope. However, from these investigations it cannot be excluded with certainty, that there are some thylakoids disrupted in the preparation and therefore the inside surfaces exposed and accessible to antibodies in the suspension.

Further experiments were carried out with lamellar systems isolated in sucrose solutions and disrupted. Both treatments with the high pressure in a French press²² and ultrasonic treatment led to a suspension of particles, which was homogeneous in the light microscope and could be agglutinated directly by sulpholipid antisera.

All agglutination experiments were carried out with sulpholipid antisera obtained by injection of

sulpholipid adsorbed to methylated BSA and with antisera obtained by injection of sulpholipid absorbed together with lecithin to methylated BSA. Equal results were obtained with both antisera.

Discussion

Chloroplasts isolated in sucrose solutions loose most of their stroma proteins when repeatedly washed with water, while retaining a morphologically intact lamellar system¹⁷. These lamellar systems are not agglutinated by antibodies against sulpholipid but the antibodies will be adsorbed specifically. The lamellar systems incubated with the antibodies, agglutinate when postincubated with the antigen or the rabbit-γ-globulin antiserum. This indicates that determinants of sulpholipid are accessible for the antibodies, but do not permit a direct agglutination. Such a behaviour can be explained if there are pro-

²¹ A. KAHN and D. VON WETTSTEIN, *J. Ultrastr. Res.* **5**, 557 [1961]; G. JAKOBI and E. PERNER, *Flora* **150**, 209 [1961]; E. PERNER, *Planta* **66**, 44 [1965]; T. E. WEIER, C. R. STOCKING, C. E. BRACKER, and E. B. RISLEY, *Amer. J. Bot.* **52**, 339 [1965].

²² K. L. SIMPSON, A. W. WILSON, E. BURTON, T. O. M. NAKAJAMA, and C. O. CHICHESTER, *J. of Bacteriol.* **86**, 1126 [1963].

trusions or depressions on the surface of the thylakoids and the antigens are present in these deepenings.

In our chloroplast lamellar system preparations we cannot exclude the presence of the outer membrane of the chloroplast, even though they lose most of their stroma. The stroma could be lost by perforations of the outer membrane. The pieces of the membrane residing on the surface of the lamellar system can also hinder the direct agglutination. But considering the thickness of the outer membrane it is very unlikely that a simple mixed antigen reaction would suffice for an indirect agglutination of the lamellar system.

The indirect agglutination of the lamellar system with the antibodies against ferredoxin-NADP⁺ reductase³ has been shown to be due to protrusions present on the surface of the lamellar system. These protrusions have been suggested to be the coupling factor molecules since they are washed away by EDTA treatment^{19, 23}.

In the case of sulpholipid antibodies the lamellar systems even after EDTA treatment show no direct agglutination. It is reasonable to assume that the sulpholipid molecules are located at the bottom of depressions present on the thylakoid surface. Isolated lamellar systems after disruption with ultrasonication or french press result in particles which exhibit a direct agglutination with the sulpholipid antibodies. A preparation of broken chloroplasts²⁰ also agglutinate when incubated with sulpholipid

antisera. By mechanical disruption the inside of thylakoids will be accessible for antibodies. In such preparations a direct agglutination is observed and it can therefore be assumed that sulpholipid molecules are also located on the inner side of the thylakoid membrane. However we cannot exclude with certainty that the natural distribution of the sulpholipid molecules are disturbed or the outer surface of the thylakoids is changed during mechanical disruption of chloroplasts. There is no experimental evidence to show disorganization of sulpholipid molecules or an alteration of the outer thylakoids surface during such a treatment. On the other hand electron microscopic observations of mechanically disrupted plastids, which have capacity of a direct agglutination, contain fragments of broken thylakoids²⁴.

Thus it is concluded, that the sulpholipid is located inside the thylakoids, but at least some sulphoquinovosyl residue are orientated against the outer protein layer and accessible to antibodies through some kind of spaces between the protein. Therefore the result with sulpholipid antibodies are not in contradiction to the thylakoid model proposed by MENKE and KREUTZ²⁵.

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²³ V. K. VAMBUTAS and E. RACKER, *J. Biol. Chem.* **240**, 2660 [1965].

²⁴ W. MENKE, unpublished.

²⁵ W. KREUTZ and W. MENKE, *Z. Naturforsch.* **17b**, 675 [1962]; *W. KREUTZ, Z. Naturforsch.* **18b**, 1098 [1963]; and **19b**, 441 [1964]; W. MENKE, in: *Photosynthesis Mechanisms in green Plants*, Publ. **1145**, Natl. Acad. Sciences, Natl. Res. Council 1963, p. 537; W. MENKE, in: *Biochemistry of chloroplasts*, Vol. **I**, (ed. T. W. GOODWIN) Acad. Press, London and New York 1967, p. 3.