Binding of Antibodies onto the Thylakoid Membrane
II. Distribution of Lipids and Proteins at the Outer Surface of the Thylakoid Membrane

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The number of antibody molecules which stroma-freed chloroplasts can bind out of the monospecific antisera to monogalactosyl diglyceride, tri- and digalactosyl diglyceride, sulfoquinovosyl diglyceride, phosphatidyl glycerol, sitosterol, plastoquinone, lutein and neoxanthin was determined. This number was compared to the number of antibody molecules which stroma-freed chloroplasts can maximally bind. The result is that the antibodies to the individual lipids cover at most 17 per cent of the accessible thylakoid membrane surface.

From a serum which contains both antibodies to the proteins and lipids of the thylakoid membrane, not more antibody molecules are bound than from a serum to the proteins. This means that antibodies to proteins are able to cover up the entire accessible surface of the thylakoids whereas a mixture of antibodies to the lipids, listed above, cover only one forth of the surface. Consequently, antibodies which are bound to proteins can cover up the lipid areas entirely and in turn antibodies which are bound to lipids cover up parts of the protein areas. From this it follows that the portion of the surface, which is made up by lipids must be considerably smaller than 24 per cent. Furthermore, it follows from these experiments that the lipid areas are small and that lipids probably only fill up the gaps between the protein molecules.

In the first communication [1] we reported on the number of antibody molecules which stroma-freed chloroplasts of *Antirrhinum majus* can maximally bind. It was shown that chloroplasts are able to bind from a serum that contains both antibodies to proteins and lipids, the same amount of antibodies as from an antiserum that contains only protein antibodies. From this it follows that the outer surface of the thylakoid membrane can be made up of lipids only to a lesser extent. On the other hand it was observed that antibodies to lipids are bound by stroma-freed chloroplasts in an appreciable amount [1, 2]. Furthermore, we were able to show that some of these antisera inhibit photosynthetic electron transport of chloroplasts at distinct sites [3–5]. In the following it is investigated to what degree the outer surface of the thylakoid membrane is composed of lipids.

Materials and Methods

1) Preparation of the antisera: The antisera to the lipids and pigments listed in Table I were obtained as described earlier by immunization of rabbits [2–4, 6–8]. For the immunization the lipids were adsorbed onto methylated bovine serum albumin, after having verified their purity by thin layer chromatography and spectroscopical methods. The antiserum to sitosterol was prepared in the same way (Radunz, unpublished).

2) Binding of antibodies onto stroma-freed chloroplasts: The isolation of stroma-freed chloroplasts has been described by Menke and Kreutz [9]. Also we have already reported in detail on the binding of antibodies onto chloroplasts as well as on the nitrogen determination and the calculation of the amount of antibodies bound [1]. The number of antibody molecules was determined according to

$$\frac{\text{Loschmid's number} \times \text{amount of protein bound (g)}}{\text{Molecular weight of the antibodies}}.$$

For the molecular weight of the antibodies (immunoglobulines of rabbits) we used 1.5×10^5. As the serum to trigalactosyl diglyceride exhibits a cross reaction with digalactosyl diglyceride [2] the number of antibodies bound out of this serum was referred to the total concentration of the tri- and digalactolipid (Table II and Fig. 1).

Results and Discussion

In the first publication on this subject [1] it was shown that the concentration dependence of the antibody binding onto stroma-freed chloroplasts yields

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Table I. Maximal binding of antibodies to lipids and xanthophylls by stroma-freed chloroplasts of *Antirrhinum majus*.

Antiserum	Reaction of antibodies with stroma-freed chloroplasts	g Antibodies bound/ g stroma-freed chloroplasts
Monogalactosyl diglyceride	bivalent	0.16 ± 0.02
Tri- and Digalactosyl diglyceride	bivalent	0.17 ± 0.01
Sulfoquinovosyl diglyceride	monovalent	0.12 ± 0.01
Phosphatidyl glycerol	monovalent	0.13 ± 0.01
Sitosterol	monovalent	0.07 ± 0.02
Plastoquinone	monovalent	0.06 ± 0.02
Lutein	monovalent	0.09 ± 0.02
Neoxanthin	monovalent	0.08 ± 0.02

A bivalent reaction of antibodies with chloroplasts leads to an agglutination whereas a monovalent reaction yields the agglutination only after addition of anti-γ-globuline. The values of column 3 for monogalactosyl diglyceride [1], tri- and digalactosyl diglyceride [2], sulfoquinovosyl diglyceride and phosphatidyl glycerol [1] have been taken from previous publications.

saturation curves. In Table I the saturation values for the lipid antibodies are compiled. All listed antisera are monospecific. In addition the table contains the information whether the antibodies react with stroma-freed chloroplasts mono- or bivalently. However, for the following discussion this difference is of no importance. As the antibodies in the region of saturation react monovalently no agglutination occurs. The preparation of the antisera to plastoquinone [3], lutein [3], and neoxanthin [4], as well as their influence on photosynthetic electron transport were reported earlier. The preparation of the antiserum to sitosterol was carried out in a similar way as that of the other lipid antisera. Details on the specificity of this antiserum will be reported elsewhere.

In Table II the number of antibody molecules bound by stroma-freed chloroplasts is compared with the number of antigen molecules that occur in the thylakoid membrane [10–12]. The result is that the numbers of the maximally bound antibody molecules differ only by a factor of 4 whereas the number of lipid molecules occurring in the lipid mixture varies by a factor of 40. If one plots for the respective lipids the number of antigen molecules occurring in 1 g of chloroplasts against the number of antibody molecules maximally bound by the same amount of chloroplasts, then Fig. 1 is obtained. It is seen, that if the lipid concentration in chloroplasts exceeds a certain value, the amount of antibodies bound does not depend anymore on the number of antigen molecules present. From this result we infer that only a certain amount of lipid antibodies is bound out of monospecific antisera. As we had determined earlier that 1 g stroma-freed chloroplasts can bind 42×10^{17} antibody molecules to lipids and proteins and as we find here that those preparations bind maximally only 7×10^{17} antibody molecules to lipids it is concluded that maximally 17% of the thylakoid surface can be covered up by antibodies to the single components of the lipid mixture. Consequently, maximally 17% of the thylakoid membrane surface consists of lipid molecules. This conclusion is only correct if the

Table II. Comparison of the number of lipid and xantophyll molecules present in stroma-freed chloroplasts with the number of bound antibodies.

Antiserum	Antigen molecules/ g stroma-freed chloroplasts	Antibody molecules bound/ g stroma-freed chloroplasts
Monogalactosyl diglyceride	850×10^{17}	6×10^{17}
Tri- and Digalactosyl diglyceride	680×10^{17}	7×10^{17}
Sulfoquinovosyl diglyceride	140×10^{17}	5×10^{17}
Phosphatidyl glycerol	150×10^{17}	5×10^{17}
Sitosterol	30×10^{17}	3×10^{17}
Plastoquinone	20×10^{17}	2×10^{17}
Lutein	94×10^{17}	4×10^{17}
Neoxanthin	30×10^{17}	3×10^{17}

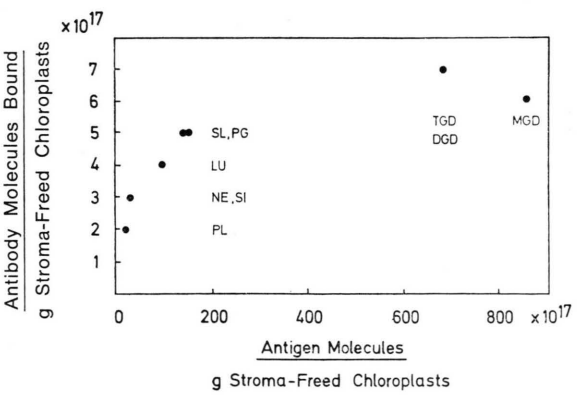

Fig. 1. Dependence of the antibody molecules bound on the number of lipid and xantophyll molecules occurring in 1 g stroma-freed chloroplasts of *Antirrhinum majus*. MGD, monogalactosyl diglyceride; TGD and DGD, tri- and digalactosyl diglyceride; SL, sulfoquinovosyl diglyceride; PG, phosphatidyl glycerol; SI, sitosterol; PL, plastoquinone; Lu, lutein; NE, neoxanthin.

lipid mixture at the surface has the same composition as the mixture of extracted lipids. As this is not necessarily true [13], a mixture of antisera to the lipids listed in Table I was prepared. From this complex antiserum 10×10^{17} antibody molecules were bound by 1 g stroma-freed chloroplasts. Hence, the surface which can be covered up by lipid antibodies should be 24%. However, from this result it cannot be derived that the outer surface of the thylakoid membrane consists by one forth out of lipids. If one keeps in mind that from an antiserum to stroma-freed chloroplasts which contains antibodies to proteins and lipids maximally the same amount of antibodies is bound as from an antiserum that contains only antibodies to protein, it follows that the antibodies adsorbed onto proteins cover up the lipid areas entirely [1]. Hence, due to the size of the antibody molecules and their hindering shape the antibodies which are adsorbed at the border of the protein areas also cover up a considerable portion of the adjacent lipid areas, *i. e.*, as much that between them no lipid antibody can find the necessary space anymore. Correspondingly, as lipid antibodies also cover up a portion of the protein areas, the portion of the thylakoid membrane surface that is made up by lipids is smaller than the area covered up by lipid antibodies. However, a calculation of the overlapping appears to be difficult as the orientation of the adsorbed antibodies is unknown. We think that the error is not too big if one assumes that $10-15$ per cent of the thylakoid membrane surface are made up by lipids. Moreover, it follows from the experiments, that the respective lipid areas are only small in diameter. From this it appears reasonable to assume that the lipids in the outer surface of the thylakoid membrane fill up the gaps between protein molecules.

According to small angle X-ray scattering the thylakoid membrane consists of a layer with higher and a layer with lower electron density, with the layer of higher electron density being directed towards the outside [9, 14, 15]. This observation was interpreted to mean that the outer surface consists entirely or to the larger extent of proteins, whereas the inner layer consists totally or preferentially of lipids [16, 17]. As the serological investigations have led to the result that the outer surface of the thylakoid membrane contains much more proteins than lipids [1], one can assume considering all known results that the inner surface of the thylakoid membrane consists mainly of lipids. That also the inner surface of the thylakoid membrane contains proteins follows from serological investigations by Koenig *et al.* [18]. In addition it should be noted that the results on the molecular structure of the outer surface of the thylakoid membrane, discussed above, are only valid for those regions of the thylakoid membrane surface which are accessible to antibodies. The surface of the thylakoids within the grana regions of the lamellar system may have quantitatively another composition.

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