

On a Quantitative Determination of Antibodies to Lipids and Proteins

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The amount of precipitating antibodies in monospecific lipid- and protein antisera was determined by quantitative precipitation reactions according to the methods of Heidelberger and Kendall. Antisera were obtained by immunization of rabbits. Fragments of the thylakoid membrane from *Antirrhinum* chloroplasts were used as antigens for the binding of antibodies. These fragments had a diameter of 100 Å. They were composed of 51% proteins and 39% lipids.

It was found that antisera to the lipids mono-, tri- and digalactosyl diglyceride, sulfoquinovosyl diglyceride and phosphatidyl glycerol contained 20 to 92 µg precipitating antibodies per ml serum. In antisera to the coupling factor of photophosphorylation, cytochrome f, a polypeptide with the apparent MW 24000 and a serum to the total protein of the lamellar system 23 to 99 µg were found. In chloroplast antisera the amount of all precipitating lipid- and protein antibodies amounted to 765 µg per ml. The described antisera originated from blood that had been withdrawn from the test animals 4 to 10 week after antigen injection.

In a series of publications it has been demonstrated that immunization of rabbits with lipid- and pigment-serumalbumin-emulsions resulted in antibodies not only to proteins but also to lipids [1–17] (functioning as haptens) and to pigments [18–24]. Using erythrocytes, bacteria (for reviews see H. Schmidt [25] and C. Steffen [26]) and chloroplasts from higher plants [8, 9, 11, 17, 18] as antigens, where lipids are present as lipid-protein-complexes, immunization yielded complex antisera with a relatively high concentration of antibodies to lipids. The formation of antibodies to lipids was shown by means of the passive heme agglutination test [11], precipitation reactions of lipid-protein-emulsions [8, 27] and the VDRL-Microflocculation technique [28]. The titer determinations following these techniques allowed but half-quantitative assertions about the activity of the resulting lipid antisera. In the present publication it is reported on a method permitting the quantitative determination of precipitating lipid antibodies.

The binding of antibodies to lipids and proteins to thylakoid membrane fragments from *Antirrhinum* chloroplasts was determined by quantitative precipitation reactions according to Heidelberger and Kendall [29–31]. These measurements were done in

correlation with investigations on the distribution of lipids and proteins in the thylakoid membrane [27, 32–36]. The antibody concentration can be calculated from the maximally adsorbed antibodies to membrane fragments in the region of equivalency.

Methods

1. Preparation of the antisera

The antisera in Table I were obtained according to earlier described methods by immunization of rabbits. For the production of protein antisera normally 1 mg of antigen emulsified in 1 ml Freund's adjuvant was subcutaneously injected and after 4 weeks 1 mg of antigen was intravenously injected. Blood was withdrawn from the animals 9 days after the last injection and then in intervals of 7 days as long as the serum could be shown to contain antibodies.

To obtain lipid antisera 2 mg of lipids with 1 mg of methylated bovine serum albumin emulsified in 1 ml Freund's adjuvant, were subcutaneously injected. After 4 weeks 2 mg of lipids emulsified in 1 mg of methylated bovine serum albumin in 1 ml 0.06 M phosphate buffer after Sørensen were injected every second day. Blood withdrawals, as for the protein antisera, were carried out every 7 days. All the applied lipid- and protein antisera were

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monospecific as demonstrated in earlier papers [8, 9, 11, 17, 34].

2. Quantitative precipitation of the antibodies and determination of the precipitating antibody concentration

For quantitative precipitation experiments lipid antiserum mixtures of the 4th to 8th blood withdrawal and protein antisera from the 4th to 10th withdrawal were used. Sera of the 1st to 3rd withdrawal were used for qualitative tests as described in previous papers [8, 9, 11, 17, 20, 24]. Membrane fragments of the lamellar system of *Antirrhinum chloroplasts* were used as antigens for the binding of lipid- and protein antibodies [35]. They were obtained by ultrasonic treatment of stroma-freed chloroplasts with subsequent fractionated centrifugation. The fragments had an average diameter of 100 Å and they were composed of 51% proteins and 39% lipids. Their thickness corresponded to the diameter of the thylakoid membrane. Antigenic determinants of lipids and proteins are located in such a way that they are accessible for antibodies [8, 9, 11, 17, 18, 20–24, 35–37].

Constant quantities of antiserum (Table I) were mixed with increasing amounts of antigen (membrane fragments from 1–50 µg Fig. 1) with subsequent quantitative determination of the precipitate after 16 h of reaction time following an earlier described method [35]. Fig. 1 shows the

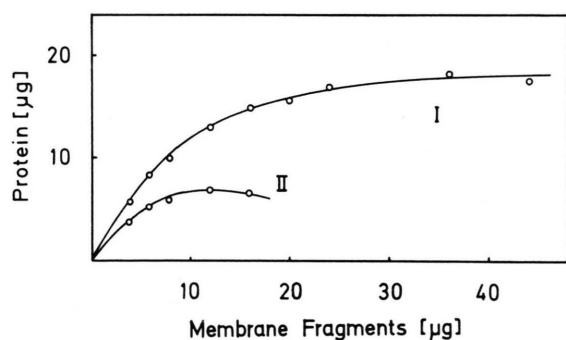


Fig. 1. Quantitative precipitation reaction of an antiserum to sulfoquinovosyl diglyceride (0.35 ml) with fragments of the thylakoid membrane from chloroplasts of *Antirrhinum majus* (4–40 µg per assay). Curve I: Protein content of the precipitate plotted as a function of the added membrane fragments. Curve II: Bound sulfoquinovosyl diglyceride antibodies plotted as a function of the added membrane fragments. The values are averages of 12 determinations.

course of the quantitative precipitation reaction of lipid- and protein antisera with membrane fragments of *Antirrhinum chloroplasts* for the sulfoquinovosyl diglyceride-antibody precipitation taken as an example. Curve I represents the dependency of the amount of protein contained in the precipitate as a function of the added membrane fragments. The slope of the curve strongly increases in the regions of antibody excess and equivalency, but it does not decrease in the region of antigen excess. In contrast to this observation, Heidelberger and Kendall [29–31] obtained curves with a maximum in the region of equivalency when they investigated precipitation reactions of soluble antigens with homologous antisera from rabbits. If one subtracts the known amount of protein of the added precipitated membrane fragments from the amount of protein of the precipitate, then the precipitated antibody amount is obtained. Plotting these values against the used amount of antigen yields curve II (Fig. 1) which goes through a maximum which is then in accordance with Heidelberger and Kendall. The maximum may then reflect the upper limit of the region of equivalency (Point of equivalency). In the region of equivalency the precipitated antigen is identical to the added one. As in this region all antibodies are precipitated as shown by precipitation reactions, the maximum of curve II represents the amount of precipitating antibodies. The results are summarized in Table I and compared to the amount of antibodies referring to 1 ml antiserum.

Furthermore the quantity of precipitating antibodies was calculated using the correlation found by Heidelberger and Kendall [29–31] as well as that by Kabat [38].

For the precipitation curve II in Fig. 1 the following equation is valid.

$$y = ax - bx^2 \quad (1)$$

with y representing the precipitated antibody quantity and x the amount of membrane fragments. In order to determine the constants, the quotients of antibodies and antigens in the precipitate were plotted as a function of the membrane fragments (Fig. 2) according to the Heidelberger-Kendall equation, which yields a straight line

$$y/x = a - bx \quad (2)$$

The ordinate section a obtained by extrapolation represents the relation antibody/antigen in the case

Table I. Content of precipitating antibodies in lipid-, protein- and chloroplast antisera.

Antiserum	Serum quantity [ml]	Amount of precipitating antibodies in the region of equivalency from Fig. 1 calculated from Eq. (4)		Concentration of precipitating antibodies per ml serum	
		[μg]	[μg]	[μg]	molecules × 10 ⁻¹²
Stroma-freed chloroplasts	0.02	15.2	15.3	765	3072
Monogalactosyl diglyceride	0.20	13.9	13.4	67	269
Tri- and digalactosyl diglyceride	0.10	9.6	9.2	92	369
Sulfoquinovosyl diglyceride	0.35	6.9	7.0	20	80
Phosphatidyl glycerol	0.30	9.3	9.1	30	121
Total protein of the thylakoid membrane	0.10	9.4	9.9	99	398
Coupling factor	0.10	7.4	7.0	70	281
Cytochrome f	0.20	4.7	4.6	23	92
Polypeptide 24 000 MW	0.10	4.3	4.5	45	181

Homologous lipid antisera were obtained by immunization of 4 rabbits. Sera from the 4th to 8th blood with-drawals were mixed and used for the precipitation reaction. Hence, data concerning the precipitating lipid antibodies are averages of 4 homologous antisera. Data of the precipitating protein- and chloroplast antisera are averages of 2 homologous antisera. These sera originated from the 4th to 10th blood withdrawal. The calculation of the antibody concentration per ml serum is based on the antibody quantities derived from Eqn. (4).

of infinite dilution of the antigen. The increase b can be calculated from the coordinate sections (Table II). The precipitated amount of antibody at the point of equivalency leads after zeroing of the derivation of Eq. (1) $y = 0 = a - 2bx_{\max}$ to

$$X_{\max} = \frac{a}{2b} \quad (3)$$

Introduction of Eq. (3) into Eq. (1) leads to

$$Y_{\max} = ax_{\max} - bx_{\max}^2 = \frac{a^2}{4b} \quad (4)$$

At the point of equivalency the relation between antibodies and antigen is constant, *i.e.* equivalent to half of the ordinate section of Eq. (2)

$$\frac{Y_{\max}}{X_{\max}} = \frac{a}{2}$$

The calculated values are with an error of $\pm 3\%$ in accordance with those taken from Fig. 1 Curve II. This method has the advantage that especially values in the region of antibody excess *i.e.* in the initial section of the curve at low antigen concentrations enter into the calculation.

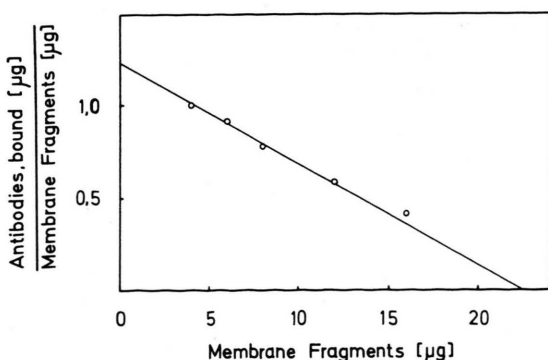


Fig. 2. Quotient of bound sulfoquinovosyl diglyceride antibodies and precipitated membrane fragments as a function of the added membrane fragments.

Results

As it can be seen from the data of Table I, the antisera to the anionic lipids contain 20–30 μg and the galactolipid antisera 67–92 μg precipitating antibodies per ml serum. If one assumes that the gammaglobuline concentration in rabbit serum amounts to 12 mg/ml [25] it follows that in the anionic lipid antisera 0.2–0.3% and in the galactolipid antisera 0.6–0.9% of the present gammaglobulins are precipitating antibodies. The antibody concentration in the protein antisera is very low too, *i.e.* 0.2–1% of the amount of gammaglobuline. The low content of precipitating antibodies can easily be ex-

Table II. Constants for the calculation of the amount of precipitating antibodies in lipid-, protein- and chloroplast antisera.

Antiserum	Constants	
	a $\frac{\mu\text{g antibodies}}{\mu\text{g antigen}}$	b $\frac{\mu\text{g antibodies}}{(\mu\text{g antigen})^2} \times 10^2$
Stroma freed chloroplasts	2.77	12.53
Monogalactosyl diglyceride	1.31	3.19
Tri- and digalactosyl diglyceride	1.06	3.05
Sulfoquinovosyl diglyceride	1.24	5.49
Phosphatidyl glycerol	1.18	3.84
Total protein of the thylakoid membrane	0.95	2.26
Coupling factor	1.00	3.55
Cytochrome f	0.40	0.87
Polypeptide 24 000 MW	1.00	5.50

plained by the fact that the applied lipid- and protein antisera are mixtures of the 4th to 8th and 4th to 10th blood withdrawal after the last intravenous injection of antigen. The antibody titer shortly after immunization of the rabbits is very high but decreases quickly with time. It was observed that bovine serum albumin (BSA) antisera of the first blood withdrawal 9 days after the booster injection contained 830 μg of precipitating antibodies corresponding to 7% of the gammaglobuline content. After the 6th blood withdrawal, *i.e.* 42 days later, however, this value had decreased to 4%. It should be noted that the BSA sera were precipitated using the identical procedure with BSA as antigen.

The highest concentration of precipitating antibodies could be found in chloroplast antisera. In comparison to the monospecific lipid- and protein antisera the antibody concentration could be shown to be 8 to 38-fold higher. This means that in these antisera 6.4% of the total amount of gammaglobulines are precipitating antibodies. These antisera to stroma-freed chloroplasts are directed to native proteins and lipid-protein complexes as well as to glycolipopeptides [39] of the thylakoid membranes *i.e.* to the largest part of the membrane components. It could be demonstrated that the antiserum reacts in precipitation reactions, double diffusion tests and immune electrophoretic assays in agarose gel with several proteins involved in the electron transport

like cytochrome f and ferredoxin-NADP reductase [34, 40, 41] as well as with coupling factor and carboxy-dismutase [34]. Furthermore, positive reactions could be observed with some water-insoluble polypeptides with apparent molecular weights between 11 000 and 66 000 (for a review, see Schmid, Menke, Radunz and Koenig [42]). Antibodies to membrane lipids like monogalactosyl diglyceride [11] tri- and digalactosyl diglyceride [17] and sulfoquinovosyl diglyceride [8] as well as to the phosphatides phosphatidyl glycerol [9], phosphatidyl cholin and phosphatidyl inositol [27] were detected by means of the passive heme agglutination test. As evidenced earlier by titer determinations the amount of precipitating lipid antibodies is 4 times higher in chloroplast antisera than in monospecific lipid antisera obtained by immunization with lipid-BSA-emulsions [8, 9, 11, 17, 22]. From this one can calculate that the mentioned lipid antibodies in chloroplast antisera can amount to 10–50% of the precipitating antibodies.

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