IMMUNOCHEMICAL ANALYSIS OF MEMBRANE VESICLES AND CHROMATOPHORES OF *RHODOPSEUDOMONAS SPHAEROIDES* BY CROSSED IMMUNOELECTROPHORESIS

Marieke G. L. ELFERINK, Klaas J. HELLINGWERF, Paul A. M. MICHELS, Hendrik G. SEŸEN+ and Wil N. KONINGS

Department of Microbiology, Biological Centre, Kerklaan 30, 9751 NN Haren and +*Department of Histology, University of Groningen, Oostersingel 69-1, 9713 EZ Groningen, The Netherlands*

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1. Introduction

Two types of vesicular membranes can be isolated from phototrophically grown *Rhodopseudomonas sphaeroides* [1]. So-called membrane vesicles are derived from the cytoplasmic membrane by osmotic lysis of lysozyme–EDTA-treated cells. Alternatively chromatophores can be derived from invaginations of the cytoplasmic membrane by French press disruption of intact cells [1]. Both preparations have been used extensively for studies on energy transduction such as light-dependent cyclic electron transfer [2,3], proton translocation [4,5], photophosphorylation [6,7] and solute transport [1,5,8].

For the interpretation of the results obtained from these studies two questions about these membrane preparations have to be answered:

1. How is the membrane oriented in these vesicular structures (with respect to the cytoplasmic membrane in intact cells)?
2. Do both membrane preparations have comparable structural and functional properties?

Qualitative information about the orientation of membrane vesicles and chromatophores has been obtained from studies on the localization of cytochrome c_2 and ATPase activity, the direction of the light-induced protonmotive force and the protonmotive force-driven accumulation of amino acids and Ca^{2+} [1,5,8]. These studies revealed that membrane vesicles are mainly right-side out while chromatophores have an inverted orientation. Quantitative information about the orientation of both membrane preparations was supplied by freeze-fracture electron microscopy [9]. Information about the chemical composition of the membranes was obtained from fatty acid analysis, determinations of bacteriochlorophyll and carotenoid content, measurements of enzyme activities and from SDS–PAA gel electrophoresis of solubilized membranes [1]. These studies indicated a large degree of similarity in the chemical composition of both preparations.

Recently crossed immunoelectrophoresis has been introduced as an extremely powerful tool for quantitative analysis of membrane immunogens and their orientation in the plane of the membrane [10–12]. This method has been applied to membrane vesicles and chromatophores from *Rps. sphaeroides* and the results of this study are presented here. We found that $\geqslant 75\%$ of the membrane vesicles have a right-side out orientation and that $> 90\%$ of the chromatophores are inside out. Furthermore, the immunoprecipitation patterns from chromatophores and vesicles are very similar indicating that the majority of the proteins present in both preparations is the same.

Abbreviations: CIE, crossed immunoelectrophoresis; SDS, sodium dodecyl sulphate

2. Materials and methods

2.1. *Cell growth and preparation of membrane vesicles and chromatophores*

Rhodopseudomonas sphaeroides strain 2.4.1. was

grown anaerobically in the light in 3 l flasks as in [1]. Membrane vesicles and chromatophores were isolated as in [1], the only modification being that during the isolation of the membrane vesicles potassium ascorbate and dichlorophenolindophenol were omitted from the incubation media.

2.2. *Preparation of Triton X-100 extracts for crossed immunoelectrophoresis analysis*

Suspensions of membrane vesicles were centrifuged at 48 000 × *g* for 30 min at 4°C and chromatophores at 140 000 × *g* for 120 min at 4°C. The pellets were resuspended in 50 mM Tris–Cl (pH 8.6) containing 5% (v/v) Triton X-100 and 5 mM ethylenediaminetetraacetic acid (EDTA) to ~15 mg protein/ml final conc. and incubated for 5 h at 25°C. After centrifugation the supernatants, containing ~9 mg protein/ml for the vesicle suspension and 12 mg protein/ml for the chromatophore suspension, were stored in 50 μl aliquots in liquid nitrogen.

2.3. *Isolation of anti-vesicle and anti-chromatophore immunoglobulins*

Three Chinchilla giganta rabbits (4–5 kg) were used for the production of each immunoglobulin preparation [10]. Suspensions of membrane vesicles (3 mg protein/ml) or chromatophores (7 mg protein/ml) were mixed with equal volumes of complete Freund's adjuvant for first course immunizations and with incomplete Freund's adjuvant for subsequent immunizations. The first course immunizations were performed by 10 intracutaneous injections of 0.1 ml in the dorsal area 3 times in a period of a month. Subsequent immunizations were given in 1–2 months intervals by intramuscular injections of 1 ml. Blood (30 ml) was taken after 10 and 20 days, via the marginal ear vein. Sera from 6 consecutive bleedings were pooled (~ 300 ml) and immunoglobulins were isolated as in [13]. The immunoglobulin fraction was concentrated with a diaflow PM 30 filter (Amicon, Lexington, MA) to ~100 mg protein/ml. The immunoglobulins were subsequently dialysed against 0.1 M NaCl containing 15 mM NaN_3 and finally stored at –20°C.

2.4. *Crossed immunoelectrophoresis*

Crossed immunoelectrophoresis of membrane preparations solubilized with Triton X-100 was performed as in [11]. Electrophoresis of proteins was performed in 1% agarose (Miles Labs, Elkhart, IN) on immunoplates of 5 × 5 cm in a watercooled electrophoresis chamber (v. Holm-Nielsen, Copenhagen) in the first dimension at 2.5 V/cm for 2.5 h and in the second dimension at 1.5 V/cm for 16–18 h. Proteins in the immunoplates were stained with Coomassie brilliant blue R 250 (Sigma, St Louis, MO). Zymogram staining techniques were used to detect immunoprecipitates of the following enzymes: D-lactate dehydrogenase; 6-phosphogluconate dehydrogenase; NADH dehydrogenase; ATP phosphohydrolase (ATPase); L-malate dehydrogenase; glucose-6-phosphate dehydrogenase; glycerol-3-phosphate dehydrogenase; succinate dehydrogenase; acid and alkaline phosphatase [11,14–16]. The peak areas of individual immunoprecipitates were determined using a Hewlett Packard integrator after 10-fold optical enlargement of the peak area.

2.5. *Absorption experiments*

Absorption experiments of anti-vesicle and anti-chromatophore immunoglobulins were performed with intact or solubilized membrane vesicles and chromatophores [10]. The membrane vesicle and chromatophore pellets were resuspended to 4 mg protein/ml final conc. in 50 mM Tris–HCl (pH 8.6) supplemented with 1 mM $MgSO_4$ or with 5 mM EDTA and 5% (v/v) Triton X-100. The samples containing Triton X-100 were incubated for 4 h at 25°C while the $MgSO_4$ containing samples were kept at 4°C for the same period. Absorption experiments were performed by adding 0.5 ml immunoglobulin solution (containing 57 mg anti-vesicle immunoglobulin or 48 mg anti-chromatophore immunoglobulin) to 0–0.75 ml intact membrane vesicle or 0–0.25 ml solubilized membrane vesicle or chromatophore suspensions. Tris–HCl buffer (50 mM, pH 8.6) was added to give 1.25 ml final vol. The suspensions were incubated with frequent shaking for 1 h at 20°C. Membrane vesicles and chromatophores were subsequently removed by centrifugation (see above) and the supernatant fractions containing the residual immunoglobulins were stored at –20°C. For each CIE experiment 250 μl was incorporated in the second-dimension agarose gel. An analogous procedure was used for the absorption experiment with intact cells.

2.6. *Analytical procedures*

Protein was determined by a modification of the method in [17] eliminating interference by Triton

3. Results and discussion

3.1. *CIE reference patterns*

Crossed immunoelectrophoresis of Triton X-100–EDTA extracts of membrane vesicles and chromatophores of *Rps. sphaeroides* yields immunoprecipitate patterns as shown in fig.1. An analysis of several hundreds of immunoplates run at various antigen (20–60 μg protein) and immunoglobulin (5–30 mg protein) concentrations yielded composite immunoprecipitate patterns as shown in fig.2A for membrane vesicles and fig.2B for chromatophores. In the immunoprecipitates stained with Coomassie brilliant blue at least 52 discrete precipitation lines could be detected in the membrane vesicle extracts while 59 discrete rockets were found in chromatophore extracts. Most of the precipitation lines are symmetrical but some asymmetrical precipitation lines are observed (see, e.g., line 33 in membrane vesicles and line 46 and 37 in chromatophores). Between 60–80% of the membrane proteins were dissolved in Triton X-100. In immunoplates of both preparations little precipitation was observed around the application spot indicating that the solubilized membrane proteins show little tendency for aggregation.

3.2. *Cross reaction patterns*

More information [1] about the similarity in protein composition of membrane vesicles and chromatophores was obtained by cross-reacting membrane vesicles with chromatophore antibodies (fig.3A) and chromatophores with membrane vesicle antibodies (fig.3B). In these precipitation patterns the majority of the lines observed in fig.1 are present. Therefore the antigenic composition of the membrane vesicles

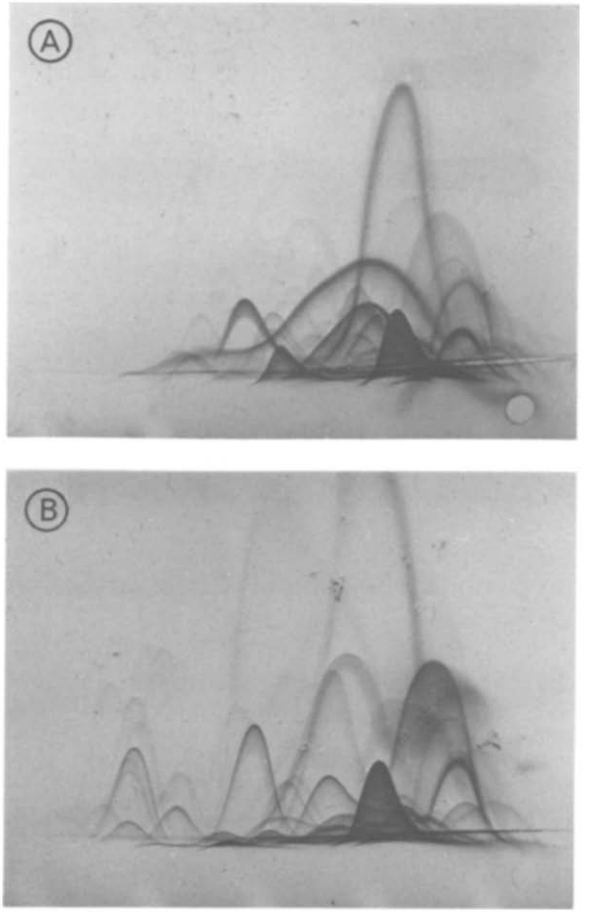
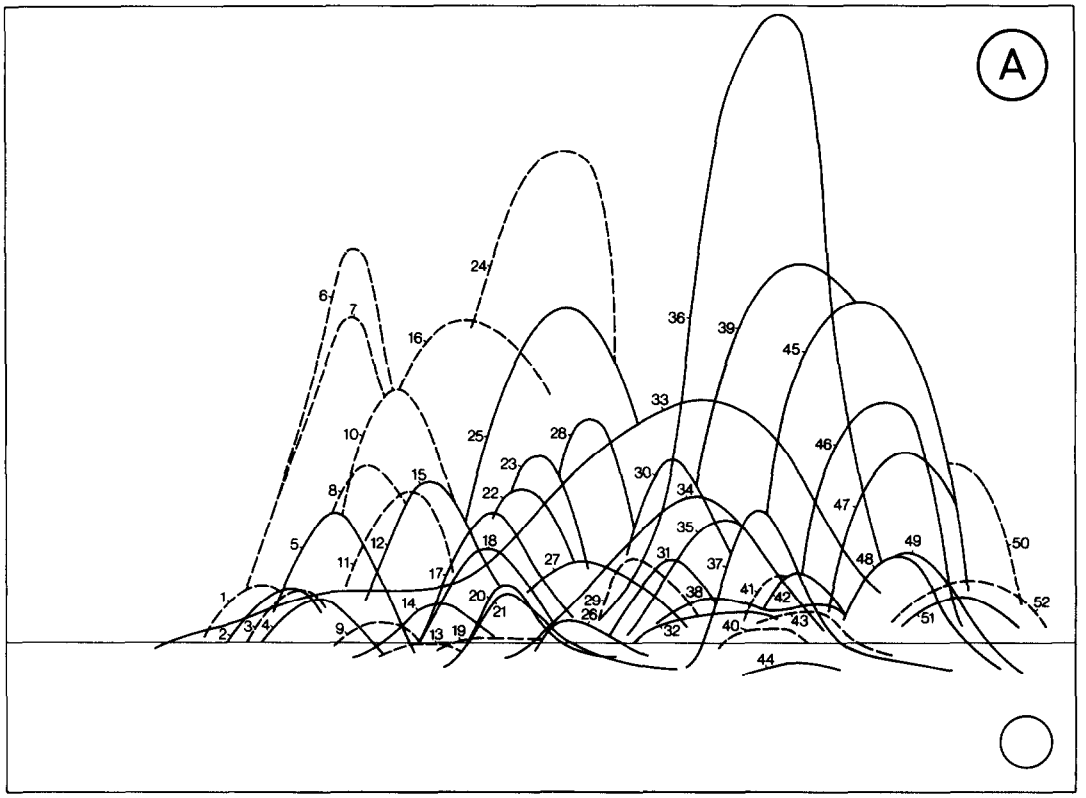
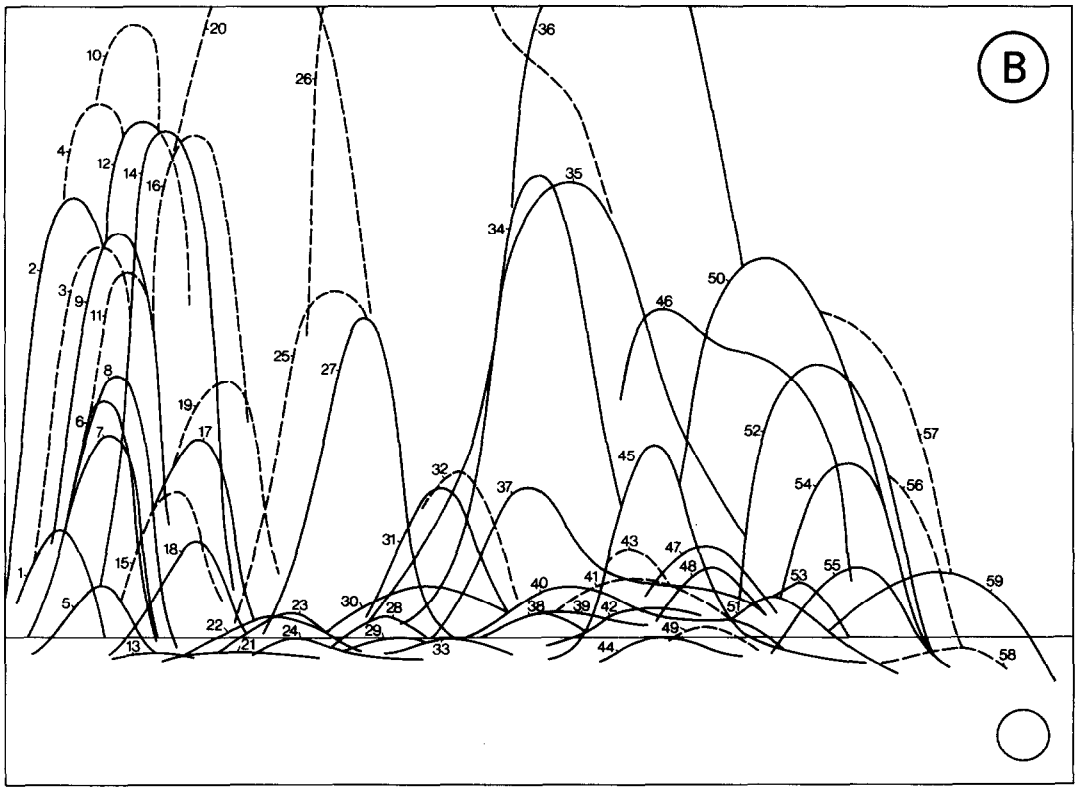


Fig.1. Typical crossed immunoelectropherograms of Triton X-100 extracts of membrane vesicles (fig.1A) and chromatophores (fig.1B) of *Rps. sphaeroides*. Electrophoresis in the first dimension was in the direction of the abscissa. Per assay 5 μl containing 45 μg membrane vesicle protein or 60 μg chromatophore protein dissolved in 5% Triton X-100 was applied on the application spot. Electrophoresis in the second dimension was in the direction of the ordinate in 1% agarose containing 22 mg immunoglobulins for membrane vesicles and 20 mg immunoglobulins for chromatophores. After electrophoresis the immunoplates were stained with Coomassie brilliant blue as in section 2. In all experiments the anode was at the left and at the top of the figures.

Fig.2. Schematic representation of crossed immunoelectropherograms of membrane vesicles (A) and chromatophores (B) deduced from electropherograms in which various concentrations of immunoglobulins were used. The broken lines represent weakly stained precipitation lines.


A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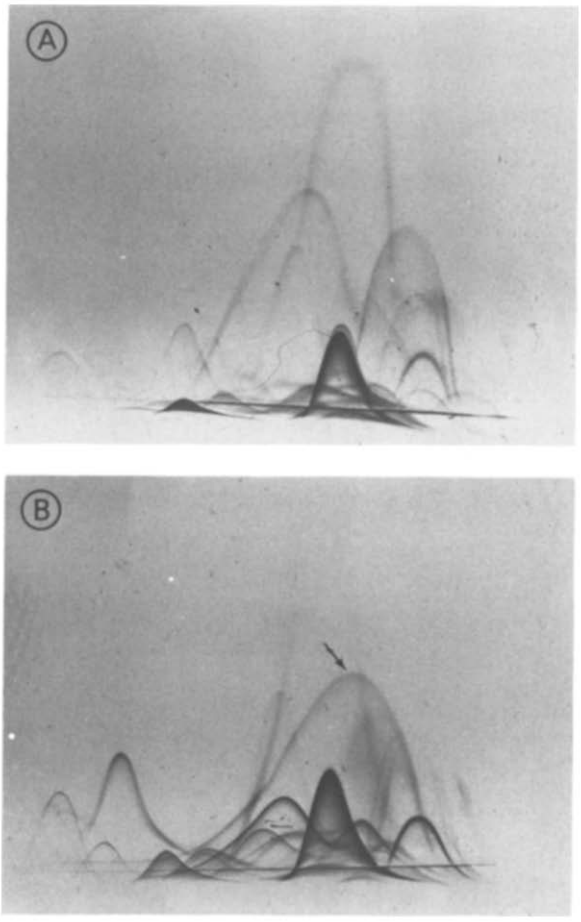

Fig.3. Cross-reaction electropherograms of membrane vesicle antigens against anti-chromatophore immunoglobulins (A) and chromatophore antigens against anti-membrane vesicle immunoglobulins (B). The experimental conditions are described in section 2 and in the legend to fig.1. The amount of chromatophore antigen applied was 60 μg and of membrane vesicle antigen 45 μg. The immunoplates contained 17 mg anti-vesicle immunoglobulin or 14 mg anti-chromatophore immunoglobulin.

and chromatophores must be very similar. This even holds for the antigen of band 33 in the membrane vesicle pattern (fig.2A). The antibody against this antigen can be removed from the membrane vesicle immunoglobulins by adsorbing with intact cells, indicating that this antigen is a component of the outer membrane (see below). The same precipitation line (indicated by an arrow in fig.3B) is found in the CIE patterns of chromatophore antigen against vesicle antibodies.

3.3. *Characterization of immunoprecipitates*

Information about the identity of the immunoprecipitate lines which have retained their enzymatic activity can be obtained by the zymogram staining technique [11,14–16]. Of the enzymes tested (see section 2) only enzymatical active immunoprecipitates were found of NADH dehydrogenase, succinate dehydrogenase, L-malate dehydrogenase and ATPase. The zymogram staining technique appears to be more sensitive than staining with Coomassie brilliant blue because not every enzymatically active immunoprecipitate could be related to a corresponding precipitation line with Coomassie brilliant blue. NADH dehydrogenase activity in membrane vesicles (fig.4A) and chromatophores (fig.4B) is found in 4 precipitation lines. These lines correspond to the Coomassie brilliant blue precipitation lines of the membrane vesicle reference pattern (fig.2A) no 41 and 42 (the other two lines were not detected with Coomassie brilliant blue staining) and of the chromatophore reference pattern (fig.2B) no 33,42,44 and 48. Succinate dehydrogenase activity is found (fig.4c and fig.4d) in one precipitation line (fig.2A, band 38 and fig.2B, band 40) which consists of two fused lines indicating that the two components have the same antigenic sites. L-Malate dehydrogenase activity is detected in one small line in membrane vesicles and in chromatophores but both lines were not detected by Coomassie brilliant blue staining (data not shown). ATPase activity was present in one single peak in both membrane vesicles (fig.4E) and chromatophores (fig.4F). These peaks correspond to the heavy precipitation bands 37 for membrane vesicles (fig.2A) and 45 for chromatophores (fig.2B). It has been mentioned above that in addition precipitation line 33 of membrane vesicles (fig.2A) could be identified as an outer membrane component since this band was removed by absorbing anti-vesicle immunoglobulins with intact cells. The enzyme activities found in these membrane preparations were, besides several other enzyme activities, also detected in CIE immunoplates of *Escherichia coli* and *Micrococcus lysodeikticus* membrane vesicles [11,12]. In contrast to the observation made in membrane preparations of *Rps. sphaeroides* NADH dehydrogenase

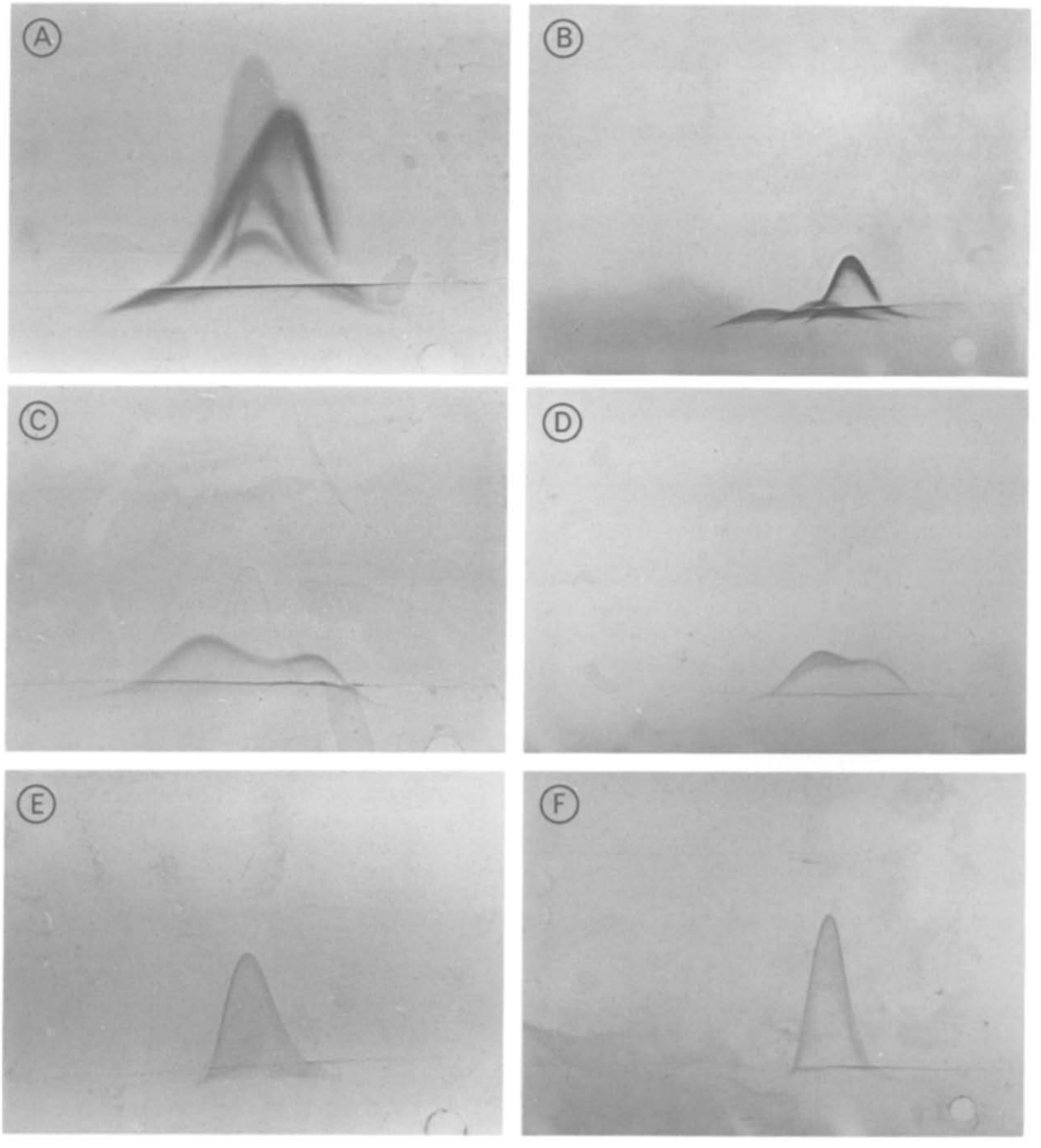

Fig.4. Identification of the immunoprecipitates of ATPase, succinate dehydrogenase and NADH dehydrogenase by zymogram staining in crossed immunoelectropherograms of membrane vesicles and chromatophores. A,C,E: Zymogram staining in CI electropherograms of membrane vesicles. B,D,F: Zymogram staining in CI electropherograms of chromatophores. A,B: NADH dehydrogenase. C,D: succinate dehydrogenase, E,F: ATPase.

activity was in *E. coli* only present in two precipitation lines and succinate dehydrogenase in one symmetrical peak. ATPase activity was, however, just like in *Rps. sphaeroides*, present in one symmetrical precipitation line.

3.4. *Orientation of the membranes*

Quantitative information about the orientation of membranes in membrane vesicles and chromatophores can be obtained by CIE. This method was first applied for *E. coli* membrane vesicles in [10]. Anti-membrane vesicle immunoglobulins or anti-chromatophore immunoglobulins are titrated with increasing amounts of intact or solubilized membrane vesicles or chromatophores, respectively. After removal of the immunoprecipitate by centrifugation CIE is performed with the remaining antibodies in the supernatant. In this way the expression of ATPase, succinate dehydrogenase and NADH dehydrogenase was quantified. Figure 5A shows the results of an absorption experiment for ATPase activity in which the reciprocal of the area of the immunoprecipitate containing ATPase activity ($1/A$) is plotted as a function of the quantity of intact or solubilized vesicles used during immunoadsorption (v). In fig.5B a similar absorption experiment performed with intact or solubilized chromatophores is shown.

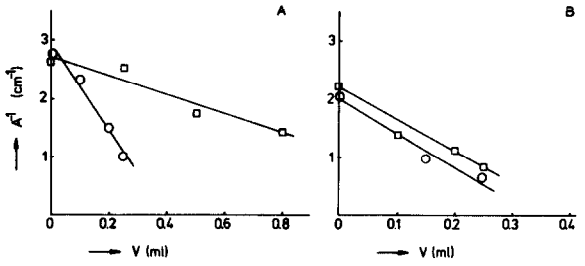

Fig.5. Peak areas of the immunoprecipitates of ATPase activity after immunoabsorption of immunoglobulins with various concentrations of membrane antigens. In A immunoabsorption was performed with 57 mg anti-membrane vesicle immunoglobulins and v ml (4 mg protein/ml) of intact vesicle suspension (□) or membrane vesicles solubilized in 5% Triton X-100 (○). In B a similar experiment was performed with 48 mg anti-chromatophore immunoglobulins and v ml (4 mg protein/ml) intact chromatophores (□) or chromatophores solubilized in 5% Triton X-100 (○). The precipitation lines of ATPase are shown in fig.2 (line 37 for membrane vesicles and 45 for chromatophores).

Linear relationships between $1/A$ and v were found if 1 ml anti-vesicle immunoglobulins was titrated with

Table 1
Percentage of membrane-bound antigens exposed at the outer surface in membrane vesicles and chromatophores of *Rps. sphaeroides*

Antigen	Membrane vesicles		Chromatophores	
	Precipitation line no.[a]	% exposed at outer surface[b]	Precipitation line no.[a]	% exposed at outer surface[b]
ATPase	37	25–32	45	94–94
Succinate dehydrogenase	38	18–21	40	129–100
NADH dehydrogenase	42	22–39	48	84–106

[a] The numbers correspond to the numbers in the reference crossed immunoelectropherograms shown in fig.2

[b] The percentages of the enzymes exposed to the outer surface were calculated from data as presented in fig.5 on the assumption that 100% of the enzymes are exposed in the Triton X-100 solubilisate

The slope of the curves was determined using a least-squares fit to the experimental data. The correlation coefficient of the resulting straight lines ranged between 0.94 and 0.99. The two values given for each enzyme are the results of two independent determinations with the use of the same antibody and antigen preparation

0–6 mg intact membrane vesicle protein or 0–2 mg solubilized membrane vesicle protein or if 1 ml anti-chromatophore immunoglobulins was titrated with 0–2 mg intact or solubilized chromatophore protein. These data already indicate that a significant difference exists in the expression of the NADH dehydrogenase, succinate dehydrogenase and ATPase antigens in intact and solubilized membrane vesicles but not in intact and solubilized chromatophores. The fraction of ATPase, succinate dehydrogenase and NADH dehydrogenase which is accessible from the outer surface of the membrane was calculated from the results obtained by these absorption experiments (table 1). In membrane vesicles and chromatophores, respectively, about 25% and 95% of these enzymes are attainable for the antibodies. The deviation of these values from 0% and 100%, respectively, can be caused by mis-orientation of sealed vesicles, scrambling or unsealed membrane sheets. In intact cells these enzymes are most likely located at the inner surface of the cytoplasmic membrane. These data therefore indicate that the orientation of $\geqslant$ 75% of the membrane vesicles is right-side out while the orientation of the chromatophores is essentially all inside out. These results are consistent with results obtained with other procedures [1,5,9] and especially with freeze-fracture studies of membrane vesicles and chromatophores from *Rps. sphaeroides*. It has been suggested that the fraction of membrane vesicles which have a right-side out orientation is higher when cells are grown under high light intensities [9].The illumination conditions employed were certainly not optimal at the time of harvesting. A higher fraction of right-side out membrane vesicles might be obtained when the cells are grown under more optimal illumination conditions.

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