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Electrophoretic analysis, labeling and isolation of *Chlamydomonas reinhardtii* flagellum membrane proteins

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Abstract

SDS-polyacrylamide electrophoretic patterns of *Chlamydomonas* flagellum membrane proteins displayed 6 fractions, 3 PAS-positive among them. The surface radiolabeling of the flagellum membrane suggested an outer surface exposure of fraction '5', and internal localization of fractions '4' and '6'. Application of SDS-polyacrylamide gel electrophoresis and radiolabeled membranes allowed to isolate individual membrane polypeptides.

INTRODUCTION

The biflagellate, single-celled alga *Chlamydomonas reinhardtii*, is known to have several adventages for the study of cell differentiation (Jones, 1970; Sikorski, 1978). The flagellum membrane is covered with thin hairlike structures called mastigoneme (Ringo, 1967), which were found to be composed of a single high molecular weight glycoprotein (Witman et al., 1972). According to Witman et al. (1972) and Snell (1976), purified flagellum membranes give in SDS-polyacrylamide gel electrophoresis one major and one or two minor protein bands. The major band is also a glycoprotein. In this report methods for isolation as well as some properties of *Chlamydomonas renhardtii* flagellum membranes such as: SDS-polyacrylamide gel electrophoretic analysis, surface labeling of membrane polypeptides and isolation of individual polypeptide fractions are described.

MATERIALS AND METHODS

Chlamydomonas strain 89 was used. Cultures were carried out according to Kates and Jones (1964). Cultures were stirred and bubbled with 5% CO_2 in air. Cells were grown for 7-8 days to the

density of 2-4×10⁶ cells per ml in automatically controlled temperature (25°), synchronized on a 12-hours light, 12 hours dark cycle, and were harvested after four hours in light by continuous flow centrifugation at 20°C at 7000 r.p.m. (RC-2B Sorvall, SS-34 rotor). The cells remained intact and flagellated.

Flagella and flagellum membranes were isolated according to Witman et al. (1972) with small modifications: concentration of $CaCl_2$ added after STEEP (0.15 M sucrose; 15 mM Tris; 2.5 mM disodium EDTA; 11% ethanol; 30 mM KCL) treatment of cell suspension was raised to 30 mM. Flagella were purified by additional centrifugation on 40% sucrose. In membrane isolation the dialysis period was extended to 48 hours and differential centrifugation was carried out on 45% sucrose.

Cells were stirred with one volume of cold 10 mM Tris-HCL buffer pH 7.8 and 5 volumes of cold STEEP solution for 2 minutes, then $CaCl_2$ solution was added to the final concentration of 30 mM. Cell suspension (15 ml) was overlayered on 25 ml of 25% sucrose in 10 mM Tris-HCl, pH 7.8, and centrifuged at 2000×g for 10 min. The upper and middle layers containing flagella were aspirated and centrifuged for 20 min. at 31 000×g. The flagellum pellet was suspended in 10 mM Tris-HCl, pH 7.8, overlayered on 40% sucrose in 10 mM Tris-HCl, pH 7.8, and centrifuged at 3000×g. for 10 min. The flagella were collected from the upper and middle layers by centrifugation at 31 000×g.

Isolated flagella, after extensive washing with Tris-HCl, pH 7.8, were suspended in a solution containing 0.1 mM EDTA; 0.01% 2--mercaptoethanol; 1 mM Tris, pH 8.0 and dialysed for 48 hours against the same buffer. The suspension was then overlayered on 45% sucrose in 10 mM Tris-HCl, pH 7.8, and centrifuged for 90 min. at 16 000×g. Membranes remained in the upper layer, axonemes sedimented completely, and between was the layer of membranes not completely detached from the tubular material (interface, see Table 1). The membrane layer was collected and centrifuged at 105 000×g. for 2 hours (supernatant and membrane pellet, see Table 1). Preparations for electron microscopy were negatively stained with phosphotungstic acid.

Polyacrylamide gel electrophoresis was carried out in the presence of 0.1% sodium dodecyl sulphate (SDS) according to Fairbanks et al. (1971). Electrophoresis was run in glass tubes 0.5×10 cm at 2 mA per tube for 15 min. and then at 4 mA per tube for 4 hours. Gels were stained with 0.25% Coomassie brilliant blue R-250 in 40% methanol in 10% acetic acid and destained with 40% methanol in 10% acetic acid for 6 hours, followed by 10 per cent acetic acid. Periodic acid Shiff staining procedure (PAS) was carried out according to Fairbanks et

al. (1971) including gel fixation. Material which was to be subjected to electrophoresis was solubilized (to the mentioned final concentrations) in 1% SDS; 8% sucrose; 10 mM tris HCL pH 8.0; 1 mM EDTA; 40 mM DTT and 10 μg/ml of pyronin Y for 5 min. in a boiling water bath. Stained gels were scanned at 625 nm and 550 nm with a Gilford linear transport (2410 S) unit equipped with a Gilford (2000) recorder and Beckman monochromator.

Routine SDS-polyacrylamide gel electrophoresis was used to isolate polypeptide fractions of flagellum membrane. On each gel a sample of membrane suspension labeled with ^{125}I related to 200 μg of membrane protein was applied. After electrophoresis the gels were removed from the tubes, cut into 1 mm slices and the radioactivity of each was measured. Gel slices corresponding to appropriate protein fractions were pooled and extracted with 0.1% SDS in 0.04 M Tris; 0.02 M sodium acetate; acetic acid to pH 7.4 (0.2 ml for each slice) twice for 24 hours with continuous shaking. Extracts were dialysed twice against 50% (v/v) methanol, three times against water and lyophilised.

The membrane and flagella iodination procedure was taken from Boxer et al. (1974). To the suspension of flagella (5 mg protein per ml) and flagellum membranes (5 mg/ml) in isoosmotic sodium phosphate buffer (0.155 M NaH_2PO_4 adjusted to pH 7.4 with 0.103 M Na_2HPO_4) $Na^{125}I$, apprx. 2.5×10^8 c.p.m. and 0.1 mg of lactoperoxidase per milligram of protein were added. The reaction was run at room temperature. H_2O_2 (total 600 nM) was added in 10 μl aliquots at 3 min. intervals, within one hour. After iodination the flagella were washed twice with isoosmotic buffer and once with 10 mM Tris-HCl, pH 7.8, followed by the isolation of membranes. Labeled membranes as well as those isolated from labeled flagella, were washed with 10 mM Tris-HCl, pH 7.8. Labeled material was subjected to electrophoresis (about 100 μg on each gel) and stained with Coomassie blue in standard conditions. Afterwards the gels were scanned, frozen in solid CO_2 and cut in to 1 mm slices with a Joyce Loebl Gel Slicer (The Mickle Laboratory Engineering Co. USA). Radioactivity measurements were carried out with the use of the Isodyne 1185 Automatic Gamma System (Nuclear Chicago).

Protein determinations were carried out according to the Hartree (1972) modification of Lowry et al. (1951) method. Bovine serum albumin was used as a standard. Carbohydrate content analyses were performed by the phenol method of Dubois et al. (1951) and total phosphorus by the method of Bartlett (1958). Phospholipid content was estimated by multiplying total phosphorus content by 25.

Chemicals: Acrylamide, N,N′-methylene-bis-acrylamide and TEMED (N,N,N′,N′-tetramethylenediamine) were purchased from BioRad. Lactoperoxidase (40-50 units per milligram) and Tris (hydroxymethyl)-amino-

methane were from Sigma. $Na^{125}I$ (566 mCi per ml) was obtained from New England Nuclear. The other chemicals were from Fisher or Mallinckrodt and were of analytical grade.

RESULTS AND DISCUSSION

An increase of $CaCl_2$ concentration up to 30 mM allowed to avoid flagellum membrane distorsion during the isolation, which was observed when 15 mM $CaCl_2$ was used (Photos 1 and 2).

The amount of isolated flagella estimated as protein was about 0.6 mg per liter of culture.

It was found in electron microscope observations that a 16 hours dialysis period was to short (at least for strain 89) — the membranes were not completely detached from the axonemes. Axonemes, even from flagella isolated in the presence of 15 mM $CaCl_2$ and washed extensively, contained PAS-positive components which were not observed in those obtained after 48 hours dialysis.

Extension of the dialysis period provided complete detachment of membranes. Both membrane preparations contained no tubular structures, and showed no differences in electron microscope examination (Photo 3), as well as in SDS-polyacrylamide gel electrophoresis.

In Table 1 the contribution of flagella fractions obtained by differential centrifugation of flagella dialysates (16 and 48 hours dialysis) is shown. The data presented there show that the yield of membranes obtained through 48 hours dialysis was at least two-fold higher.

Table 1

Flagellum membranes isolation — total protein content in flagellar fractions obtained by differential centrifugation of dialysed flagellar suspensions

Fraction	Method I per cent of total flagellar protein	Method II per cent of total flagellar protein
Dialysed flagella	100	100
Interface	20.5	10.0
Axonemes	46.9	49.2
Supernatant	23.7	19.8
Membrane pellet	8.9	21.0

Flagellar preparations were dialysed 16 (Method I) or 48 (Method II) hours against 0.1 mM EDTA; 0.01% 2-mercaptoethanol in 0.1 mM Tris, pH 8.0. The dialysate was submitted to differential centrifugation on 45% sucrose in 10 mM Tris-HCl, pH 7.8 for 3 hours at 16000 × g. Three fractions were obtained: membranes 'interface' and axoneme pellet. The fraction containing membranes was centrifuged for two hours at 105000 × g. The data concern strain 89 vegetative flagella.

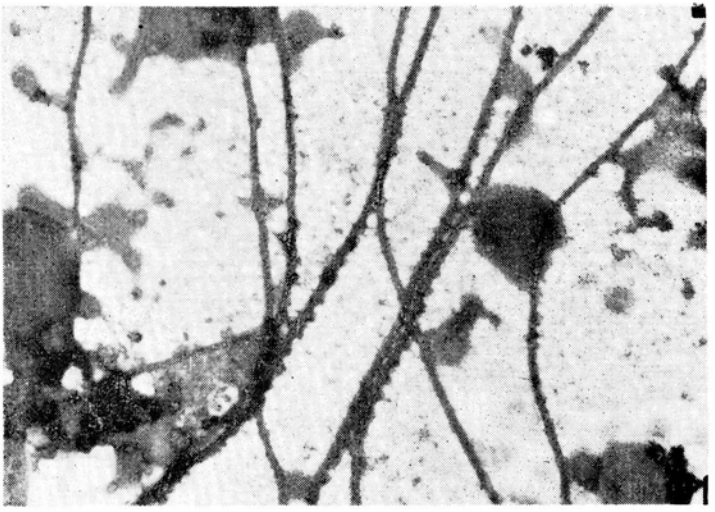

Photo 1. *Chlamydomonas* flagella isolated according to Witman et al. (1972); × 15 000, negative staining.

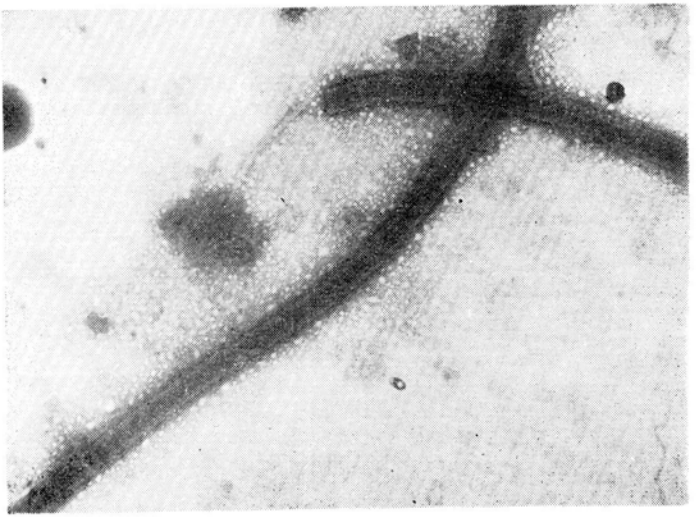

Photo 2. *Chlamydomonas* flagella isolated in the presence of 0.03 M $CaCl_2$; × 40 000, negative staining.

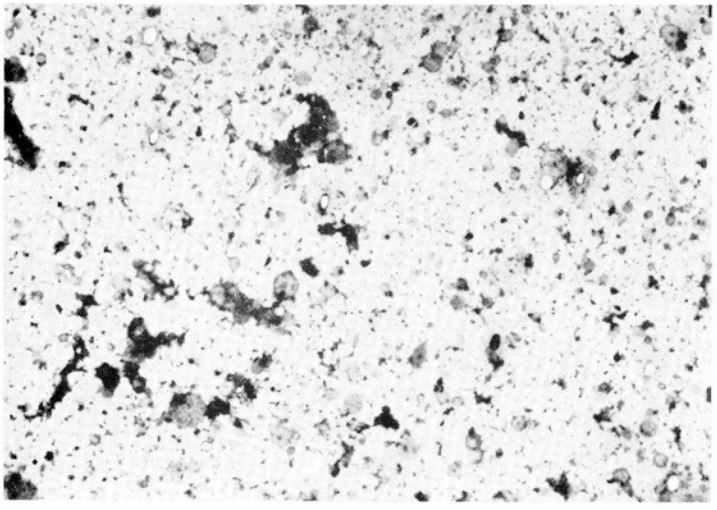

Photo 3. *Chlamydomonas* flagellum membranes isolated after 48 hours dialysis; × 25 000, negative staining.

Purified membranes were found to contain 54.1% of protein, 33.8% of carbohydrates and 12.1% of phospholipids.

Electrophoretic patterns of total flagella proteins obtained in the presence of 0.1% SDS in 5.6% polyacrylamide gel displayed ten and in 7.0% gel twelve polypeptide bands (Fig. 1). In both gel concentrations

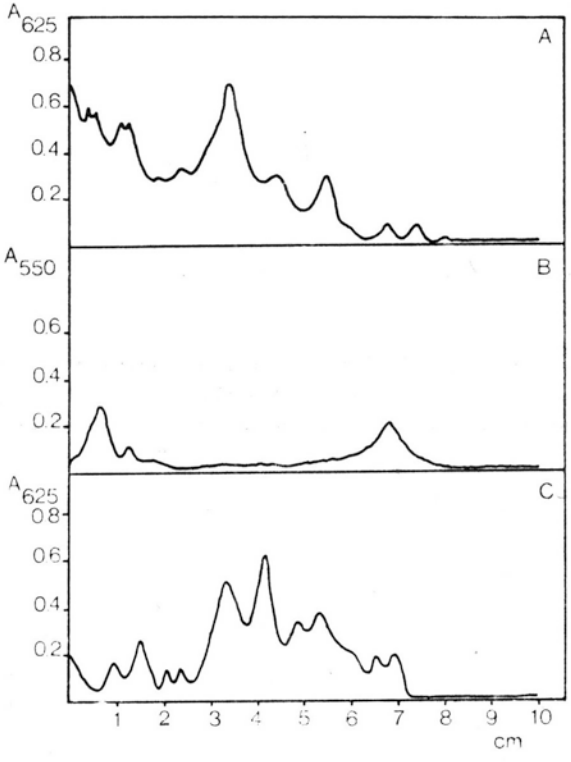

Fig. 1. Polyacrylamide gel electrophoresis of total flagellum proteins of vegetative cells, strain 89 in the presence of 0.1% SDS.

a — gel 7.0%, Coomassie blue staining, 100 μg of protein; b — gel 7.0%, PAS-staining, 100 μg of protein; c — gel 5.6%, Coomassie blue staining, 100 μg of protein.

three PAS-positve components were found. The axoneme fraction in 7.0% gel electrophoresis displayed two main tubulin bands and one minor of high molecular weight — probably aggregated tubulin, and in 5.6% gel only one major band (Fig. 2).

Electrophoretic patterns of flagellum membrane proteins obtained in 5.6% gel (Fig. 3) displayed six protein bands, including three PAS--positive components. The PAS-positive fraction '2' stained with Coomassie blue very weakly or was not stained at all.

Freshly prepared flagella were used to label proteins accessible from the external surface of the membrane, and membrane preparations to label both their sides. Both membrane preparations were subjected to

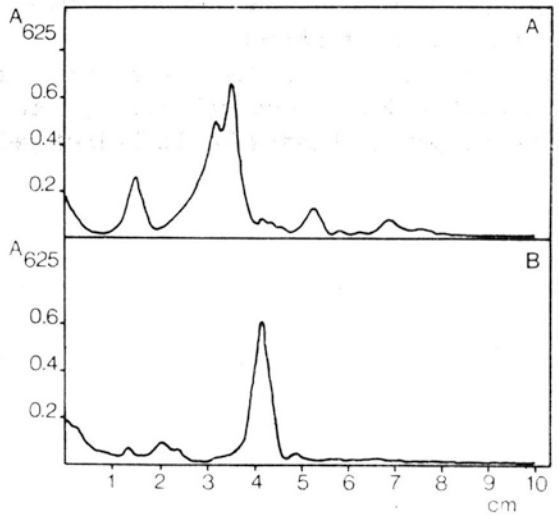

Fig. 2. SDS-polyacrylamide gel electrophoresis of strain 89 vegetative flagellum axoneme proteins.

a — gel 7.0%, Coomassie blue staining, 100 μg of protein; b — gel 5.6%, Coomassie blue staining, 100 μg of protein.

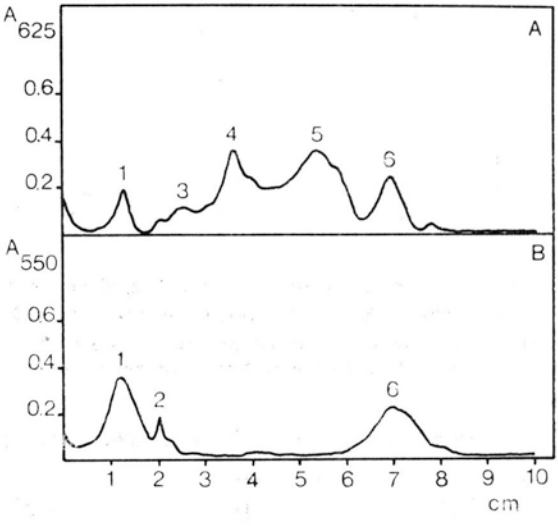

Fig. 3. SDS-polyacrylamide gel (5.6%) electrophoresis of strain 89 vegetative flagellum membrane proteins.

a — Coomassie blue staining, 100 μg of protein; b — PAS staining, 50 μg of protein.

electrophoresis in standard conditions (Fig. 4). Results of a similar experiment where the axoneme fraction of labeled flagella was used to test the impermeability of membranes to lactoperoxidase are shown in Fig. 4c.

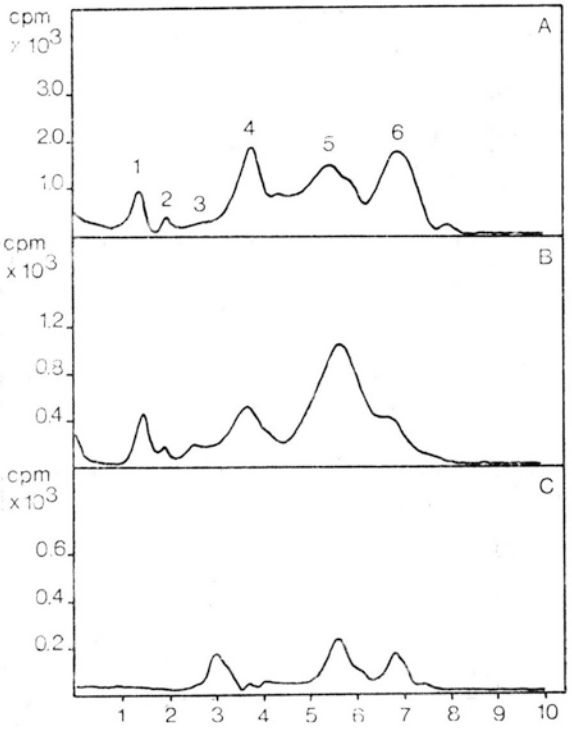

Fig. 4. SDS-polyacrylamide gel electrophoresis of strain 89 vegetative flagellum membranes labeled with ^{125}I. Radioactivity profiles.

a — isolated membrane preparation labeled, 100 μg of protein, 1.3×10^5 c.p.m.; b — membranes obtained from labeled flagella, 100 μg of protein, 2.5×10^4 c.p.m.; c — axoneme fraction obtained from labeled flagella, 100 μg of protein.

In the lactoperoxidase system almost all the flagellum membrane polypeptide fractions (with the exception of fraction '3' which is always distincly stained with Coomassie blue) can be labeled with ^{125}I. Fraction '2' which stains for protein very weakly shows a significant peak in radioactive profile at the position of glycoprotein '2'.

Comparison of radioactive profiles of membrane proteins labeled after membrane isolation and obtained from labeled flagella indicates the differences connected mainly with the amount of label in fractions '4', '5' and '6'. Fractions '4' and '6' bear relatively less label in the case of intact labeled flagella, what can suggest their localization at the internal axonemal surface. Polypeptide(s) '5' is labeled in this case more intensely, so an external, easily accessible to lactoperoxidase, localization is suggested.

The radioactive profiles obtained after electrophoresis of labeled membranes were similar to the stained and fixed ones (Fig. 4a). After extraction of pooled gel slices containing appropriate membrane polypeptide fractions 50-70% of radioactivity was recovered in the extracts.

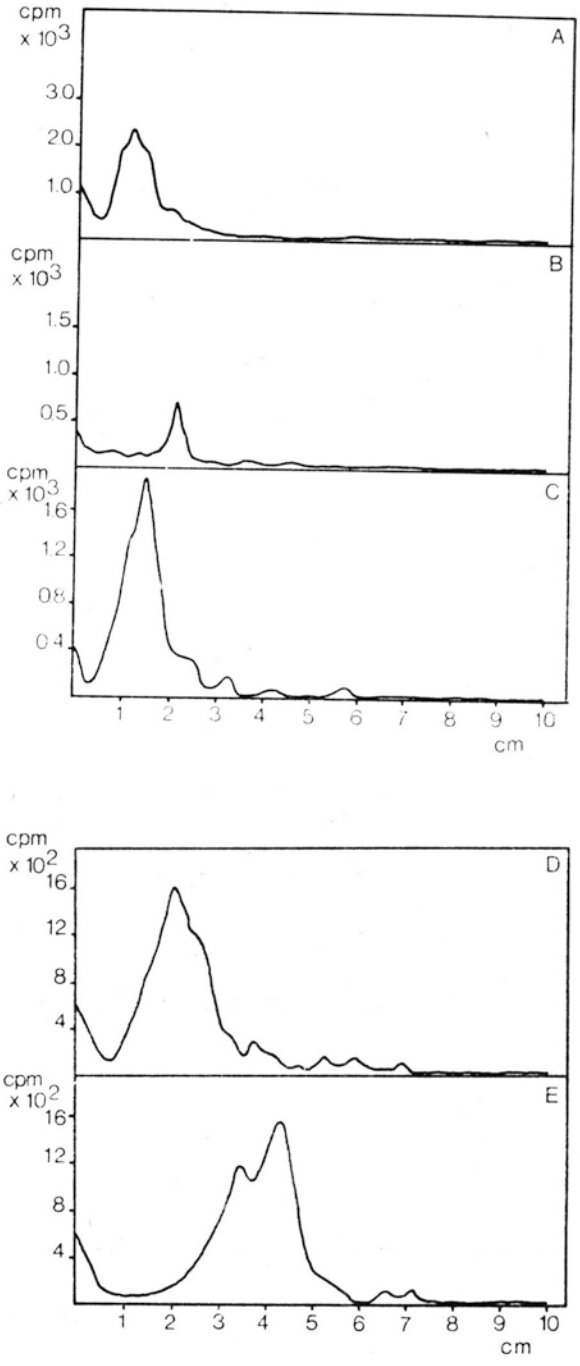

Fig. 5. SDS-polyacrylamide gel electrophoresis of isolated protein fractions of strain 89 vegetative flagellum membranes. Radioactivity profiles.

a and b — gel 5.6%, fractions no. 1, and no. 2 respectively; c, d, e — gel 7.5%, fractions no. 3, 4 and 5 respectively.

Fixed and stained with Coomassie blue gels yielded only 30-50% of radioactivity.

The radioactive tracing patterns obtained after re-electrophoresis of isolated fractions '1' and '2' in 5.6% gel and of fractions '3', '4', '5' in 7.5% gel are presented in Fig. 5. It can be seen from the presented data that isolated fractions are only slightly contaminated reciprocally and all, with the exception of fraction '5', display single peaks. Fraction '5' shows two well separated peaks.

Although the purity of the isolated membrane preparations has been well documented, the electrophoretic results differ from similar reports on flagellum membranes — Witman et al. (1972), Bergman et al. (1975), Snell (1976). Fractions '1' and '2' are the same in all cited reports, but the number of other fractions is different among them. The results of Snell (1976), who observed in the 'gamone' preparation 6-7 protein fractions seem to be in good agreement with those obtained here. Apart from one report on *Paramecium* cilia (Hansma and Kung, 1975), that small number of polypeptides in biological membranes is rather rare, e.g. Stubbaiah and Thompson (1974) observed at least 4 protein bands in the *Tetrahymena* ciliary membrane, and Chua and Bennoun (1975) as many as 33 polypeptide chains in *Chlamydomonas* thylacoid membranes. In the most extensively studied erythrocyte membranes there are at least 20 polypeptide fractions (Juliano, 1973). The differences in the number of electrophoretic fractions might be due to the different solubilization methods, the amount of material applied, and staining procedures. The lactoperoxidase system used for labeling flagellum proteins is an attempt to determine their exposure to the external flagellar surface. The results presented here suggest that polypeptides of fraction '5' or one of them are exposed to this surface. It should be noted, that tubulin in leaky flagella and in isolated axonemes was easily labeled with ^{125}I. An unexpected result is the unchanged radioactivity pattern of fraction '2' — which is supposed to be the main mastigoneme component. Probably its high carbohydrate content interferes with radiolabeling in native flagella. To sum up, the obtained results are in good agreement with the generally accepted model of membrane protein organization (Singer and Nicolson, 1972). It should be noted that no extrinsic proteins in the flagellum membrane have been observed so far. Despite attempts, membrane preparations containing more protein fractions (shorter dialysis periods) and/or less (dialysis of membrane preparations against 0.02 M EDTA pH 8.5) were not obtained.

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Elektroforeza, znakowanie i izolacja białek błon wici Chlamydomonas reinhardtii

Streszczenie

W elektroforezie w żelu poliakryloamidowym w obecności SDS białka błon wici *Chlamydomonas* wykazują obecność 6 frakcji, w tym 3 dające pozytywną reakcję z odczynnikiem Shiffa.

Znakowanie powierzchniowe J^{125} białek wici wskazuje na powierzchniową ekspozycję frakcji '5', a wewnętrzną aksonemalną lokalizację frakcji '4' i '6'.

Stosując znakowane J^{125} błony wici wyizolowano poszczególne frakcje białkowe w elektroforezie w żelu poliakryloamidowym.