The three cortical membranes of the gregarines (parasitic protozoa)

Characterization of the membrane proteins of *Gregarina blaberae**

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Gregarines, which are parasitic protozoa living in invertebrates, possess a cortical structure specific to their vegetative stage: namely two additional cytomembranes are lying just under the plasma membrane. This cortical complex has been isolated by centrifugation on discontinuous sucrose gradients and characterized chemically. Its integrity was tested by electron microscopy. Ghost proteins were resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. About 30 polypeptides of mol.wt. 15 000–300 000 were present in this fraction and four glycoproteins were detected after periodate/Schiff staining. Ten major proteins were labelled after lactoperoxidase-catalysed iodination. The GP₂ glycoprotein (41 000–49 000 apparent mol.wt.) appears to be a major component of the cell surface. Effects of trypsin and Pronase digestion on ghosts and cells were monitored by gel electrophoresis and by electron microscopy. Ghosts treated with low trypsin or Pronase concentrations (10–25 µg/ml) became drastically disorganized; many proteins were vigorously attacked in comparison with those of control ghosts. Variations in proteinase-sensitivity of proteins are pointed out. The GP₃ glycoprotein (130 000–160 000 apparent mol.wt.) seemed to be the only glycoprotein released from the cell surface by trypsin. Whole cells treated under the same conditions or with higher proteinase concentrations (up to 1 mg/ml) do not exhibit morphological modifications of the cell surface; furthermore, no discernible cleavage of membrane proteins was indicated by electrophoretograms. It is postulated that cell-surface proteins are protected by the dense carbohydrate cell coat. By using various different methods (change of ionic strength, detergent, denaturing agent, labelling experiment) it was possible to localize several major proteins within the protozoan cortical membranes.

Understanding of biological-membrane molecular organization requires information on the location of the membrane components. Singer & Nicolson (1972) have proposed a classification of membrane proteins based on the way these proteins can be removed from the membrane. Some proteins can be extracted by gentle procedures. Thus the cytochrome *c* of mitochondrial membrane can be extracted by high salt concentration (Dickerson *et*

al., 1971) and spectrin, a protein of the erythrocyte membrane, has been removed by chelating agents (Marchesi & Steers, 1968). On the other hand, other proteins require more drastic treatment, such as with detergents or protein denaturants, to dissociate them from membranes. The glycophorin of the erythrocyte has been isolated by using lithium di-iodosalicylate (Segrest *et al.*, 1972); Lauf & Poulik (1968) have used urea to solubilize proteins from human erythrocyte membranes. During last decade, numerous experiments on membrane components have been performed by using proteolytic digestions and labelling methods associated with gel electrophoresis. For example, the effects of different proteinases on the erythrocyte plasma membrane have been extensively studied by using freeze-fracture (Yamanaka & Deamer, 1976), or SDS/

* This paper is part II of a series; part I is 'Ultrastructural organization of *Gregarina blaberae*' and is as yet unpublished.

Abbreviations used: PAS₁, PAS₂, PAS₃, PAS₄, glycoconjugate bands detected by periodate/Schiff staining of the polyacrylamide-gel slabs; SDS, sodium dodecyl sulphate; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK').

polyacrylamide-gel electrophoresis (Bender *et al.*, 1971; Steck *et al.*, 1971; Triplett & Carraway, 1972; Yamanaka & Deamer, 1976). Since cell-surface glycoproteins and glycolipids are thought to be involved in cell-cell interactions and in cell-differentiation processes, labelling methods were proposed to localize either the proteins or glycoconjugates on the outer surface of the plasma membrane. The lactoperoxidase-catalysed radioiodination procedure (Phillips & Morrison, 1971) and the galactose oxidase procedure (Gahmberg & Hakomori, 1973) were used to label proteins and glycoconjugates respectively.

The action of phospholipases also afforded information on the topological arrangement of lipids in the membranes (Roelefsen *et al.*, 1974; Bevers *et al.*, 1979). In a same way, glycosidases are also suitable tools commonly used to study cell-surface glycoconjugates (Flowers & Sharon, 1979). Most investigations have been performed with sialidases, which are more readily available in purified form than other glycosidases.

Sporozoa, including gregarines, coccidia and *Plasmodium* (the malarial parasite), possess a cortical structure that is specific to their vegetative stage, namely two inner cytomembranes lying under the plasma membrane. The functions of these three cortical membranes are still unknown, but it can be postulated that they have played a fundamental part in the diversification of cell surfaces in the Sporozoa and that they have contributed to parasitic adaptations. The aim of the present work was to study the organization of a Sporozoon cell surface by using digestions with proteinases, changes in ionic strength, denaturing agents such as urea and radioiodination experiments. For this purpose, large-sized cells that can be obtained in sufficient amounts are particularly suitable. *Gregarina blaberae* therefore appears to be a better tool for such a study than small-sized cells such as those of *Plasmodium*.

Materials and methods

Cells were harvested from the midgut of the cockroach *Blaberus craniifer* and washed repeatedly with the following saline solution: 155.8 mM-NaCl/1.8 mM- Na_2HPO_4 /0.2 mM- NaH_2PO_4 /3.1 mM-KCl/1.8 mM- CaCl_2 (Spira *et al.*, 1969). Control washing was performed with the saline solution containing 100 μM -phenylmethanesulphonyl fluoride as proteinase inhibitor and 0.02% NaN_3 to prevent bacterial growth. In some experiments phosphate-buffered saline was used: 140 mM-NaCl/2.7 mM-KCl/0.9 mM- CaCl_2 /0.5 mM- MgCl_2 /8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.2.

Ghost isolation

Ghost isolation was performed as previously

described (Philippe *et al.*, 1979). Briefly, cells (1 vol.) were homogenized in 7 vol. of 50 mM-Tris/HCl/5 mM- MgCl_2 buffer, pH 7.4, containing 8% sucrose (w/w). All the operations were performed at 4°C. The homogenate was centrifuged at 10 000 g (Sorvall RC5, HB 4 rotor) for 30 min on a 45%/63% (w/w) discontinuous sucrose gradient. A ghost fraction was obtained at the 45%/63% interface. Pelleted ghosts were resuspended and homogenized in the previous 8%-sucrose/buffer and centrifuged again at 15 000 g for 30 min on a 45%/52% (w/w) discontinuous sucrose gradient. Sedimented ghosts were further purified by equilibrium centrifugation at 200 000 g (Beckman, SW 41 rotor) for 16 h on a 25–55% (w/w) continuous sucrose gradient.

Analytical procedures

Total lipids from whole cells and ghosts were extracted by the method of Bligh & Dyer (1959). Phospholipids were determined by their inorganic-phosphorus content as described by Weil-Malherbe & Green (1951). The Courchainé method for cholesterol determination (Courchainé *et al.*, 1959) was modified to detect 1 μg of cholesterol. The Folin method of Lowry *et al.* (1951) was used for protein determination. Carbohydrate content was determined by the g.l.c. procedure of Zanetta *et al.* (1972).

Preparation of materials for electron microscopy

Cells or ghosts were fixed with 5% glutaraldehyde in a 100 mM-cacodylate buffer, pH 7.4 at 4°C for 2 h. They were washed in the same buffer and post-fixed with 1% osmium tetroxide for 45 min. Then they were embedded in Araldite as described by Luft (1961). Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and observed with an Hitachi HU 11 Cs or a JEOL 100 C electron microscope.

Electrophoresis

Polyacrylamide-gel electrophoresis was performed on a 6–20% (w/v) gradient gel slab containing 0.1% SDS, with a 3% (w/v) polyacrylamide stacking gel and the Tris/glycine buffer system described by Laemmli (1970). Electrophoresis were performed for 16 h at 100 V constant voltage. Samples were dissolved in 1% (w/v) SDS/1 mM-EDTA/1% (w/v) β -mercaptoethanol/50 mM-Tris/maleate (pH 8.0)/10% (w/v) glycerol, by heating at 100°C for 5 min. The Geiger & Bessman (1972) method was used for determination of proteins in the presence of 2-mercaptoethanol.

The following proteins (purchased from Pharmacia Fine Chemicals) were used as molecular-weight markers: thyroglobulin, mol.wt. 330 000; phosphorylase b, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase,

30000; α -lactalbumin, 14400. Proteins were stained by using a solution of Coomassie Blue. Glycoproteins were detected by the periodate staining procedure of Fairbanks *et al.* (1971). For comparative purposes, identical amounts of protein were sometimes loaded on to the gel slabs.

Gels were scanned at 546 nm for Coomassie Blue staining and at 510 nm for periodate/Schiff staining.

Elution of ghost proteins by change of the ionic strength

Low-salt treatment. Ghosts were incubated with 0.1 mM-EDTA in 5 mM-sodium phosphate buffer, pH 8.0, for 30 min at 37°C. They were then centrifuged at 100 000 g (Beckman 70.1 Ti rotor) for 30 min. The pellet and the freeze-dried supernatant were subjected to electrophoresis.

High-salt treatment. Ghosts in 5 mM-sodium phosphate buffer, pH 8.0, were diluted with 1 M-NaCl (0.5 M-NaCl final concn.). The mixture was left for 30 min at 4°C. The salt-treated ghosts were pelleted in 30 min at 100 000 g. The supernatant was decanted and was dialysed overnight against 10 mM-Tris, pH 8.0, at 4°C. The pellet and the freeze-dried supernatant were then subjected to electrophoresis.

Extraction of ghost proteins by the combined action of deoxycholate and high ionic strength

Ghosts were homogenized in 5 mM-sodium phosphate buffer, pH 8.0, containing 2.5 mM-sodium deoxycholate and 0.5 M-NaCl. The homogenate was then stirred at 4°C for 2 h. Centrifugation and dialysis were completed as described above under 'High-salt treatment'. Both fractions (pellet and supernatant) were subjected to electrophoresis.

Elution of ghost proteins with 6 M-urea

Ghosts were extracted with 100 mM-Tris/HCl/5 mM-MgCl₂ buffer, pH 8.0, containing 6 M-urea for 1 h at 60°C. The urea-extracted ghosts were pelleted in 1 h at 100 000 g. The pellet and the supernatant were then subjected to electrophoresis.

Proteolytic treatment of cells and ghosts

Cells or ghosts were incubated with Tos-Phe-CH₂Cl-treated trypsin (Worthington, sp. activity 220 units/mg) or with proteinase from *Streptomyces griseus* (Sigma, type VI) also designated as Pronase, in 100 mM-phosphate buffer, pH 7.8, for 15 min or

1 h at 28°C. Two concentrations of enzymes were usually used: 10 and 25 μ g/ml. Higher concentrations of trypsin and Pronase (up to 1 mg/ml) were used on whole cells. The enzymic hydrolyses were stopped by using a trypsin inhibitor [1 mg of soya-bean trypsin inhibitor (Sigma)/1.4 mg of trypsin] after digestion with trypsin and 0.1 mM-phenylmethanesulphonyl fluoride after digestion with Pronase.

Digested cells or ghosts were washed three times with 100 mM-phosphate buffer, pH 7.8, and were prepared for electron-microscopic examination or were directly dissolved for electrophoresis experiments.

Lactoperoxidase-catalysed radioiodination of proteins

Cells were washed repeatedly with the saline solution described by Spira *et al.* (1969). Cells were spun down by centrifugation at 100 g, and 0.1–0.2 ml of cells were incubated with gentle stirring at room temperature, for 10 min in 1 ml of phosphate-buffered saline (see above), containing lactoperoxidase (10 μ g/ml) glucose oxidase (0.1 unit/ml), 5 μ M-glucose and 0.5 mCi of carrier-free Na¹²⁵I (The Radiochemical Centre, Amersham, Bucks., U.K.). Labelled cells were subsequently washed several times with phosphate-buffered saline before solubilization or proteolytic treatment. ¹²⁵I-labelled proteins were resolved by electrophoresis and made visible by conventional autoradiography of dried gel slabs with Kodirex X-ray film (Kodak). Autoradiograms were scanned at 546 nm.

Results

1. Ghost isolation and chemical composition

On homogenization, cells split longitudinally. Isolated ghosts appeared as envelopes or large fragments on phase microscopy (Plate 1). Morphological examination of ghosts by electron microscopy shows the presence of the three membranes of the cell cortex, suggesting its integrity. The cell coat is firmly bound to the external surface of the cell, as has been shown by cytochemical experiments (Philippe *et al.*, 1979).

Equilibrium centrifugation of the ghost fraction on a 25–55% (w/w) continuous sucrose gradient at

Table 1. *Comparative composition of freeze-dried ghosts and whole cells*

	Homogenate	Ghosts
Phospholipid phosphorus*/protein (μ mol/mg)	0.36–0.92	0.53–0.62
Cholesterol/phospholipid phosphorus* (molar ratio)	0.23–0.36	0.20–0.36

* Phospholipids were determined by assay of inorganic phosphorus.

200 000 *g* for 16 h, shows the presence of only one band corresponding to a sucrose density of 1.20–1.21. This result agrees with the homogeneity of the ghost preparation. Several enzymic activities were

Fig. 1. Lactoperoxidase-catalysed radioiodination of gregarine cell-surface proteins

Cells were labelled, then ghosts were isolated. (a–d), Ghost protein patterns. Increasing amounts of labelled proteins were loaded on to the gel slab (from left to right). Proteins were stained with Coomassie Blue. (e–h), Corresponding autoradiogram showing ^{125}I -labelled proteins. Labelling is specially intense in the PAS zones. The most prominent ^{125}I -labelled component ($\blacktriangleleft$) coincides exactly with the PAS₂ zone.

Fig. 2. Lactoperoxidase-catalysed iodination of external proteins

—, A_{546} profile of ghost proteins stained with Coomassie Blue; ----, corresponding absorbance profile of external iodinated proteins. Autoradiograms were scanned at 546 nm. Labelling is specially intense in the 41 000–49 000-mol.wt. zone, which coincides with the location of the PAS₂ component. Designated proteins are expressed in $10^{-3} \times \text{mol.wt.}$

tested and the 5'-nucleotidase activity was found not to be firmly bound to the gregarine-cell surface (result not shown).

Phospholipid/protein and cholesterol/phospholipid ratios of ghosts were compared with those of the whole homogenate (Table 1).

The variability of the phospholipid/protein ratio was found to be wide for the homogenate, but was narrow for the ghost fraction (this result will be discussed below). Cholesterol/phospholipid molar ratios are not increased in the ghost fraction.

Different determinations on freeze-dried ghost fractions indicated a 2.5% (w/w) carbohydrate content. The carbohydrate composition was found to be analogous to that previously reported (Philippe *et al.*, 1979).

2. Identification of the ghost proteins and iodinated proteins

(a) Electrophoresis of ghost proteins. The poly-

Fig. 3. Extraction of ghost proteins by various treatments: urea, ionic-strength variation and sodium deoxycholate

(a–c), 6 M-Urea extraction of the ghost proteins: (a), control (ghost proteins stained with Coomassie Blue); (b), urea-insoluble fraction (pellet of the 100 000 *g* centrifugation); (c), urea-extracted proteins (supernatant of the 100 000 *g* centrifugation). (d–g), Elution of ghost proteins by change of the ionic strength and by use of sodium deoxycholate; (d), control (ghost proteins stained with Coomassie Blue); (e), ghost proteins eluted by low-salt treatment (0.1 mM EDTA/5 mM-sodium phosphate, pH 8.0); (f), ghost proteins eluted by high-salt treatment (0.5 M-NaCl/5 mM-sodium phosphate, pH 8.0); (g), ghost proteins eluted by sodium deoxycholate and high-salt treatment (2.5 mM-deoxycholate/0.5 M-NaCl/5 mM-sodium phosphate, pH 8.0).

EXPLANATION OF PLATE 1

Phase-contrast photomicrograph of the gregarine ghost

Ghosts were obtained by centrifugation of homogenized cells on discontinuous sucrose gradients (see the Materials and methods section). The cell has disrupted longitudinally and the ghost appears as the whole envelope of the cell. Fine longitudinal striations on the ghost correspond to the cell-surface folds (magnification of the cell surface is seen in Plate 2*b*). Magnification $\times 160$.

EXPLANATION OF PLATE 2(a)

Electron micrograph showing the effect of trypsin (25 µg/ml) treatment on isolated ghosts

The ultrastructure is greatly disorganized; the plasma membrane (pm) is disrupted and the two inner cytomembranes (im) exhibit electron-dense condensations. Some membrane vesicles (mv) are seen. sf. Surface folds. Magnification $\times 50000$.

EXPLANATION OF PLATE 2(b)

Effect of a high trypsin concentration (500 µg/ml) on whole cells

No discernible modification of the cell surface is seen between trypsin-treated cells and control cells. Abbreviations: sf, surface folds; pm, plasma membrane; im, inner cytomembranes. Magnification $\times 55000$.

peptide patterns obtained by using gel electrophoresis were very reproducible. Protein patterns of ghosts isolated in the presence of phenylmethanesulphonyl fluoride and NaN_3 were identical with those obtained without inhibitors, showing that no 'artificial' proteolysis and no bacterial contamination occurred during the isolation procedure.

The ghost fraction protein profile on a 6–20% gradient gel slab exhibited about 30 bands after Coomassie Blue staining. These bands included proteins of molecular weight between 15 000 and 300 000 (Fig. 4*f*).

The periodate/Schiff staining method permits the detection of four broad zones on the ghost pattern (designated as PAS_1 – PAS_4), corresponding to the glycoproteins (Philippe *et al.*, 1979).

(b) *Lactoperoxidase-catalysed iodination of external proteins.* By using liquid-scintillation counting we have checked that labelling did not occur inside the cell during the incubation process; the total radioactivity was recovered in the ghost fraction, so it can be asserted that all labelled proteins subsequently resolved by polyacrylamide-gel electrophoresis belong to the ghost.

Correlation of ghost proteins stained by Coomassie Blue and cell-surface proteins labelled by

^{125}I radioiodination is shown in Figs. 1 and 2. Increasing amounts of solubilized ghost proteins were subjected to electrophoresis. At least ten major proteins were radioiodinated. Labelling was specially intense in the PAS_1 – PAS_4 stained zones (Figs. 1 and 2). The PAS_2 component of the glycoprotein profile coincided with the most prominent peak of ^{125}I label. Several other proteins were labelled: the 225 000-mol.wt. protein and some low-molecular-weight proteins. Additional minor components seemed to be weakly labelled and were not clearly resolved.

3. Segregation of the ghost proteins by change of the ionic environment and by denaturing agents

(a) *6 M-Urea elution of the isolated ghosts.* The urea-soluble fraction of the ghosts (supernatant of the 100 000 *g* centrifugation) is characterized by numerous protein bands that are normally present in the protein profile of untreated ghosts (compare Fig. 3*c* and Fig. 3*a*). Three major components of the urea-soluble fraction are the 97 000-, 90 000- and 52 000-dalton proteins. The 75 000-, 64 000-, 46 000-, 41 000-, 40 000-, 33 000-, 28 000- and 27 000-dalton proteins are also representative of this soluble fraction. In contrast, the 225 000- and 217 000-

Fig. 4. *Electrophoretic-protein-pattern modifications induced by trypsin treatment of isolated ghosts*

Experiments were carried out with increasing trypsin concentrations and time incubations. For comparative purpose, identical amounts of protein were loaded on to the gel slab (approx. 30 μg of protein). (a–e), Electrophoretic profiles of the glycoproteins stained by the periodic acid/Schiff method; (f–j), Coomassie Blue-stained proteins. (a) and (f), control electrophoretogram (1 h incubation in trypsin buffer); (b) and (g), 15 min treatment with trypsin (10 $\mu\text{g}/\text{ml}$); (c) and (h), 1 h treatment with trypsin (10 $\mu\text{g}/\text{ml}$); (d) and (i) 15 min treatment with trypsin (25 $\mu\text{g}/\text{ml}$); (e) and (j), 1 h treatment with trypsin (25 $\mu\text{g}/\text{ml}$). The PAS_3 glycoprotein is progressively removed; a new PAS zone appears that may coincide with the intensifying 67 000-mol.wt. protein (see arrow).

dalton proteins are exclusively present in the urea-insoluble fraction (Fig. 3b). This fraction shows also an enrichment in 161000-, 72000-, 49000- and 19000-dalton components when compared with the control.

(b) *Extraction of ghost proteins at low ionic strength.* As shown in Fig. 3(e), incubation of ghosts at low ionic strength induces the selective release of numerous proteins. The 75000-, 56000- and 48000-dalton proteins are major components of the supernatant. The 97000-, 90000-, 64000-, 41000-, 33000, 28000- and 27000-dalton components are also extracted by low-salt treatment.

(c) *Extraction of ghost proteins at elevated ionic strength.* Only few proteins are extracted by high-salt treatment (Fig. 3f). Major components are the 56000-, 48000- and 33000-dalton proteins; these proteins were found to be also released by low-salt treatment, whereas the 97000, 75000-, 64000- and 27000-dalton proteins are only partially extracted.

(d) *Extraction of ghost proteins by the simultaneous action of sodium deoxycholate and high ionic strength.* The whole proteins that are extracted by high-salt treatment (see above) are also found on this electrophoretogram (Fig. 3g). However, an additional 49000-dalton component is released by the specific action of the detergent.

4. Proteolysis experiments

(a) *Proteolysis of isolated ghosts.* A 1 h treatment of the ghosts with trypsin (25 µg/ml) disorganized the surface folds (Plate 2a). The plasma membrane was disrupted and in cross-sections the two inner cytomembranes exhibited electron-dense granule-like structures. Some membrane vesicles are seen between the folds.

Several membrane proteins were cleaved (Figs. 4f-j) mainly at the level of high-molecular-weight proteins. Additional bands, such as those corresponding to 21000-, 24000- and 67000-dalton proteins, appeared on the electrophoretograms. Some of them were further digested by trypsin (e.g., 21000- and 24000-dalton proteins).

The proteolytic sensitivity of the ghost proteins, tested with different time incubations and with two trypsin concentrations (10 and 25 µg/ml), were different, as shown on electrophoretic profiles (Figs. 4f-j). 217000-, 97000-, 75000-, 64000-, 52000-dalton proteins were very sensitive to trypsin (they completely disappeared on treatment for 15 min with 10 µg of trypsin/ml). The 161000- and 225000-dalton proteins were also rapidly digested; their complete removal was achieved with trypsin (25 µg/ml) within 60 min.

90000- and 41000-dalton proteins were less sensitive to trypsin digestion; they were slowly digested and were not completely removed after a 60-min treatment with trypsin (25 µg/ml). The 49000-dalton

protein seemed to be particularly resistant to trypsin treatment.

Observation of periodic acid/Schiff staining patterns showed that the PAS₃ glycoprotein was progressively removed on trypsin treatment of isolated ghosts (Figs. 4a-e). Correlatively, a new periodate/Schiff broad zone appeared that may coincide with the intensifying 67000-dalton component. Similar experiments on isolated ghosts using Pronase from *Streptomyces griseus* also lead to a rapid digestion of numerous proteins (Fig. 5). The results are similar to those obtained with trypsin (compare Figs. 5 and 4). Thus the 217000-, 97000-, 75000-, 64000- and 52000-dalton proteins were rapidly digested with the lower Pronase concentration. One observed difference concerns the 161000-dalton protein, which is more sensitive to Pronase digestion than to trypsin digestion [it disappears on a 15 min treatment with Pronase

Fig. 5. Electrophoretic-protein-pattern modifications induced by Pronase treatment of isolated ghosts.

For experimental details see the legend to Fig. 4. (a), Control electrophoretogram (1h incubation in Pronase buffer); (b), 15 min treatment with Pronase (10 µg/ml); (c), 1h treatment with Pronase (10 µg/ml); (d), 15 min treatment with Pronase (25 µg/ml); (e), 1h treatment with Pronase (25 µg/ml).

Fig. 6. Effects of trypsin and Pronase treatments on whole cells

Analysis was by SDS/polyacrylamide-gel electrophoresis after ghost isolation. (a–c), Effect of trypsin. Experiments were carried out with increasing trypsin concentrations. For comparative purposes, identical amounts of protein were loaded on to the gel slab (approx. $30\mu\text{g}$ of protein). Proteins were stained with Coomassie Blue. (a), Control electrophoretogram (1 h incubation in trypsin buffer); (b), 1 h treatment with trypsin ($10\mu\text{g}/\text{ml}$); (c), 1 h treatment with trypsin ($25\mu\text{g}/\text{ml}$). (d–f), Results of similar experiments using Pronase. (d), Control electrophoretogram (1 h incubation in Pronase buffer); (e), 1 h treatment with Pronase ($10\mu\text{g}/\text{ml}$); (f), 1 h treatment with Pronase ($25\mu\text{g}/\text{ml}$). (g–i), Autoradiography of cell-surface iodinated proteins after trypsin or Pronase digestion of whole cells. Cells were labelled, then they were digested with proteinases. (g), Control (1 h incubation in the proteinase buffer). (h), 1 h treatment with trypsin ($25\mu\text{g}/\text{ml}$); (i), 1 h treatment with Pronase ($25\mu\text{g}/\text{ml}$). No difference was seen between the three iodinated-protein patterns; no loss of iodinated protein occurred after trypsin or Pronase treatment of whole cells.

($10\mu\text{g}/\text{ml}$)). The 225 000-dalton protein is released by Pronase at the same rate as that observed with trypsin. The 90 000-, 72 000-, 41 000- and 28 000-dalton components, which were found to be less sensitive to trypsin than the other major ghost proteins, are also slowly digested with Pronase. The second major difference concerns the 49 000-dalton protein, which is digested by Pronase.

(b) *Proteolysis of whole cells.* Cells digested under the above conditions or with high trypsin concentrations (from $100\mu\text{g}$ to $1\text{mg}/\text{ml}$) were morphologically identical with control cells as could be judged from the appearance of the cell surface under the electron microscope (Plate 2b).

Comparative polyacrylamide-gel electrophoresis,

after treating cells with various concentrations of trypsin, showed that the protein patterns were not affected (Figs. 6a–c). Similar experiments performed with Pronase led to the same result (Figs. 6d–f). No modification of the electrophoretic protein patterns was observed when higher trypsin and Pronase concentrations ($500\mu\text{g}$ and $1\text{mg}/\text{ml}$) were used. Therefore the major proteins were not readily accessible on trypsin or Pronase treatment of whole cells.

Carbohydrate-staining profiles were quite similar for both untreated and proteinase-treated cells when these two enzymes were used (results not shown).

In order to ensure that minor surface components were not affected by proteolytic treatment of whole

cells, we first labelled the cells by using the lactoperoxidase-catalysed radioiodination system. These cells were then treated with trypsin or Pronase. After proteolytic digestion, portions of the enzyme incubation medium were tested by liquid-scintillation counting to check that no release of cell-surface-labelled material occurred during the digestion process. In a similar manner, portions of the isolated ghost fractions were also counted for radioactivity after digestion of the labelled cells. In both cases the results indicated clearly that no significant loss of labelled material occurred in proteinase-treated cells when compared with the control. Autoradiography of cell-surface labelled proteins after trypsin (25 µg/ml) or Pronase (25 µg/ml) digestion of whole cells (Fig. 6*h* and Fig. 6*i* respectively) showed that no loss of iodinated protein occurred when comparison was made with untreated cells (Fig. 6*g*).

Discussion

The isolation procedure led to an enriched ghost fraction. Only one band of material was obtained by further equilibrium centrifugation of isolated ghosts on a continuous sucrose gradient. These results agreed with a good purification of this fraction. Examination of isolated ghosts by phase-contrast and electron microscopy provided also good evidence for the purity and integrity of the ghost fraction.

The 1.20–1.21 density found for isolated ghosts was rather high compared with other isolated plasma membranes. However, Adoutte *et al.* (1980) reported a 1.15–1.20 buoyant density for the ciliary membranes isolated from *Paramecium tetraurelia*. Such high densities were generally found in plasma-membrane vesicles with a high carbohydrate content. The relative thickness of the gregarine cell coat (Schrével, 1972), as well as the characteristic folded structure of the cortex with its three membranes, might account for this high density.

Comparison of phospholipid/protein ratio and cholesterol/phospholipid ratio of ghosts with those of other cell membranes was rather difficult owing to the special ghost structure. The variability of the phospholipid/protein ratio that has been observed for the homogenate might be partially explained on the basis that gregarines were not grown on a defined medium but are parasitic organisms harvested from the host gut. It is well known that lipid synthesis in the membranes of 'non-grown' cells is not strictly regulated.

According to the ultrastructural observations it could be expected that the chemical compositions of the plasma membrane and ghost inner cyto-membranes are dissimilar; one objective of the present study was to gain knowledge about the

organization of the proteins within the ghost membranes by different and complementary methods such as digestion with proteinases, variation of the environment ionic strength, the use of denaturing agents and a labelling method.

Proteinase-digestion experiments

Proteinases are commonly used on whole cells to provide information about plasma-membrane structure (Wallach, 1972), since proteins that are removed are presumably exposed to the cell surface or held in the membrane by proteins (anchoring proteins) which are themselves exposed and susceptible to enzyme digestion. As electrophoretograms stained with Coomassie Blue indicate, it was not possible to remove major proteins from the cell surface by either trypsin or Pronase treatment on whole cells. We have checked, by using lactoperoxidase-catalysed radioiodination to label the cells, that subsequent digestion of labelled gregarines by proteinases was unable to release any minor iodinated component from the cell surface. It is postulated that proteins of the gregarine cell surface are protected by the dense carbohydrate cell coat. These cells could therefore be protected from proteolytic attack of the trypsin-like endopeptidases that are known to be present in cockroach midgut (Engelman, 1969).

In contrast, when trypsin or Pronase treatment occurred on both sides of isolated ghosts, many proteins were vigorously attacked. Variations in proteinase-sensitivity of the proteins partially depend on their accessibility in the three cortical membranes and on their composition, since the specificity of trypsin is directed towards lysine and arginine residues, whereas Pronase possesses a wider spectrum of activity.

Minor differences were found in the sensitivity of proteins to attack by trypsin or Pronase. Proteins that were rapidly digested by trypsin were usually rapidly hydrolysed by Pronase. In a same way, proteins that were less sensitive to trypsin were also slowly digested by Pronase. The only observed major difference concerns the 49 000-dalton protein.

The GP₃ glycoprotein (130 000–160 000 apparent mol.wt.) was digested by trypsin; all the other glycoproteins seemed to be resistant to trypsin treatment.

Differences between intact cells and isolated ghosts in accessibility to membrane components, such as those observed in the present study, were also found in erythrocytes by Phillips & Morrison (1971) on lactoperoxidase labelling, by Zwaal *et al.* (1971) on treatment with phospholipase C, and by Carraway *et al.* (1971) on treatment with trypsin; these authors concluded that proteins or phospholipids of the erythrocyte surface are protected by the carbohydrate part of the glycoprotein.

Localization of the proteins

The labelling method described by Phillips & Morrison (1971) is based on the ability of the lactoperoxidase to catalyse iodination of tyrosine and histidine residues of proteins. Lactoperoxidase is a high-molecular-weight protein that cannot pass through the membrane except by endocytosis (endocytosis does not occur in *Gregarina blaberae* during the labelling process). Thus only those proteins that are on the external surface of the membrane and which possess exposed tyrosine residues could react with the enzyme and consequently would be iodinated.

Application of this method on gregarines allowed detection of six major proteins and all GP₁–GP₄ glycoproteins. Labelling was particularly intense in the 41 000–49 000 mol.wt. zone of the protein profile (GP₂ zone), so it can be concluded that these four glycoconjugates are, at least in part, exposed at the outer surface of the cell and that GP₂ is a major component of the cell surface.

According to the fluid-mosaic model, membrane-

associated proteins can be classified into two groups termed peripheral proteins or extrinsic proteins and integral or intrinsic proteins (Singer & Nicolson, 1972; Vanderkooi, 1972). The peripheral proteins are easily extracted in lipid-free form from the membrane by use of chelating agents or by a change in the ionic strength of the environment.

By using 6M-urea and elevated and low ionic strength, 12 proteins were extracted from gregarine ghosts and thus might be considered as non-integrated proteins (Tables 2 and 3). Among these proteins, the 52 000-dalton protein, which is only solubilized by the urea treatment, is supposed to be a major component of the ghost-associated filaments (J. Schrével, M. Philippe & B. H. Anderton, unpublished work).

The integral proteins are tightly bound to the membrane and can only be solubilized by denaturing agents or by detergents. They have been suggested to have an amphiphilic structure (Lenard & Singer, 1966; Wallach & Zahler, 1966).

The six proteins that remained in the ghost

Table 2. *Distribution of the major external proteins and glycoproteins in the plasma membrane*

Molecular weight	Mode of association with the membrane:		Proteinase sensitivity:	
	Peripheral proteins	Integral proteins	Pronase-sensitive proteins	Trypsin-sensitive proteins
Proteins				
225 000		225 000	225 000	225 000
217 000		217 000	217 000	217 000
97 000	97 000		97 000	97 000
75 000	75 000		75 000	75 000
28 000	28 000		28 000	28 000
19 000		19 000		
Glycoproteins				
GP ₁ (12 000–15 000)				
GP ₂ (41 000–49 000)				
GP ₃ (130 000–160 000)				GP ₃
GP ₄ (>330 000)				

Table 3. *Characteristics of the non-iodinated ghost proteins*

Molecular weight	Urea-extracted proteins	Urea-insoluble proteins	Proteins eluted by low ionic strength	Proteins eluted by high ionic strength	Proteinase-sensitivity:	
					Pronase-sensitive proteins	Trypsin-sensitive proteins
161 000		161 000			161 000	161 000
90 000	90 000		90 000		90 000	90 000
72 000		72 000			72 000	72 000
64 000	64 000		64 000	64 000	64 000	64 000
56 000			56 000	56 000		
52 000	52 000				52 000	52 000
49 000		49 000			49 000	
46 000	46 000					
41 000	41 000		41 000		41 000	41 000
40 000	40 000					
33 000	33 000		33 000	33 000		
27 000	27 000		27 000	27 000		

fraction extracted by 6M-urea and were not eluted by change of the ionic strength are closely associated with the cortical membranes and should be regarded as integral membrane constituents. Among these six proteins, only the 49000-dalton component was solubilized with 2.5mM-sodium deoxycholate. This finding reflects differences in hydrophobicity between the proteins; the 49000-dalton protein is not so hydrophobically anchored in the membrane as the other integral proteins. Furthermore, this protein, which was found to be resistant to trypsin digestion, was hydrolysed by Pronase. One possible explanation of this finding might be that this protein does not possess exposed lysine or arginine residues.

The results obtained with 6M-urea and ^{125}I labelling experiments allow a more accurate segregation between proteins. Thus the 225000-, 217000- and 19000-dalton proteins might be regarded as integral proteins of the plasma membrane having a hydrophilic region protruding outside the cell, so they could be called ectoproteins according to Rothman & Lenard (1977). The 97000-, 75000- and 28000-dalton proteins are peripheral proteins of the plasma membrane exposed on the external side of the membrane. These findings would explain the particular sensitivity of the 97000- and 75000-dalton proteins to trypsin or Pronase attack.

As seen in Table 3, the other major ghost proteins could also be classified into integrated and non-integrated proteins, but their exact localization within the three cortical membranes require additional studies and new methods of investigation.

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