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MOLECULAR ORGANIZATION IN BACTERIAL CELL MEMBRANES III. COMPONENTS OF A "SOLUBLE" FRACTION OBTAINED BY *n*-BUTANOL EXTRACTION OF *STREPTOMYCES ALBUS* MEMBRANES*

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SUMMARY

We have isolated a "soluble" fraction of *Streptomyces albus* G membranes or membranes previously solubilised by sodium dodecylsulphate, using *n*-butanol extraction. Polyacrylamide gel electrophoresis in sodium dodecylsulphate of the whole membrane showed a complex protein pattern (about 20–25 bands) with two predominant groups. The "soluble" fraction represented about 25 % of the membrane protein and contained part of the major polypeptides. The yield of protein in "soluble" form decreased when membranes were suspended in water and did not significantly change if membranes were reduced with sodium dithionite and then treated with iodoacetamide. A change in relative mobility of some of these polypeptides seemed to occur with membrane delipidation. The proteins of the fraction appear to be glycoproteins as indicated by their simultaneous staining for protein and carbohydrate and the parallel sensitivity to trypsin of both stains. The apparent molecular weights by sodium dodecylsulphate gel electrophoresis of the proteins (glycoproteins) were: 63 000, 40 000 and 17 000. Similar protein patterns were obtained by extraction of the membranes with EDTA and non-ionic detergents. Lipid and nucleotide material were also found in the "soluble" fraction.

The "soluble" fraction showed by gel filtration on Sephadex G-200 the existence of different states of aggregation. These states of aggregation revealed the same electrophoretic pattern of proteins, which seemingly corresponded to that of the original fraction (i.e. three protein groups with relative mobilities 0.65, 0.80 and 1.0). Treatment of the samples under different conditions with 1 % dodecylsulphate (supplemented or not with 0.5 % β-mercaptoethanol) failed to completely dissociate the fraction as shown by Sephadex filtration.

INTRODUCTION

The properties of biological membranes could be better understood from the knowledge of the structural arrangement and properties of their components [1].

* Taken from a Thesis submitted to the University of Madrid as a partial requirement for a Ph. D. degree in Biological Sciences.

Most of the structural studies carried out so far, have been concerned with the eukaryotic plasma membrane and specially that from erythrocytes [2, 3].

Much less attention has been devoted to the bacterial plasma membrane [4]. In a previous paper [5] we studied the protein and lipid composition of the membranes of *Streptomyces albus* G and detected low levels of the enzyme activities usually associated with bacterial membranes [4]. We have since attempted the study of the protein components of these membranes under different solubilising conditions. In the present work we would like to report the effect of the treatment of the membranes of *S. albus* G with *n*-butanol with or without prior solubilisation with sodium dodecylsulphate. The protein appearing in soluble* form in the aqueous phase has been characterized by polyacrylamide gel electrophoresis in both cases and compared to that obtained by treatment with non-ionic detergents or chaotropic agents. These fractions show some of the major components of the whole membrane pattern in dodecylsulphate-gel electrophoresis. The definition of the *n*-butanol-"soluble" fraction seemed of interest for its contribution to the study of membrane protein solubilisation as well as for its possible implication in understanding the molecular organization of the whole membrane. The state of aggregation of this "soluble" fraction and the dissociative effect of sodium dodecylsulphate on it were followed by gel filtration.

MATERIALS AND METHODS

Organism

S. albus G, kindly provided by Dr J. M. Ghuysen, was grown as described [5]. All these studies were carried out with 20-h-old cells.

Membrane preparation

Membranes obtained as described previously [6] at a concentration of 3 mg protein/ml in 0.2 M sodium acetate buffer (pH 5.0) containing 30 mM EDTA were incubated with ribonuclease (50 μg/mg of protein) at 37 °C for 40 min. The membranes were then centrifuged at 27 000 × g, 20 min at 4 °C. This was the standard membrane preparation used as starting material in subsequent work.

Enzymes and chemicals

Lysozyme, trypsin, β-mercaptoethanol and iodoacetamide were purchased from Calbiochem (La Jolla, Calif., U.S.A.); ribonuclease (4 × crystallized) and anthrone from Koch-Light Labs (Colnbrook, England), and deoxyribonuclease, Triton X-100, guanidinium · HCl, bovine serum albumin and L-tryptophan from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). Orcinol and Nonidet P-42 (Shell TP.7143) were obtained from B.D.H. Chemicals (Poole, England), ATP from PL Biochemicals (Milwaukee, Wisc., U.S.A.), and EDTA and sodium dodecylsulphate from Fisher Scientific Co. (Fair Lawn, N. J., U.S.A.), coomassie brilliant blue from Schwarz/Mann (Orangeburg, N.Y., U.S.A.). Sephadex G-200 beads and blue dextran were purchased from Pharmacia Fine Chemicals (Bromma, Sweden). *n*-Butanol, ribose, sodium dithionite, fuchsin (NB), sudan III, toluidine blue, and all other chemicals were of the best quality available from Merck (Darmstad, Germany).

* We consider soluble any fraction not sedimented by a centrifugal force of 100 000 × *g* for 1 h.

Analytical procedures

Protein was determined by the method of Lowry et al. [7] using bovine serum albumin as standard and also by ultraviolet spectrophotometry at 280 nm in a Gilford 2400 spectrophotometer. In some instances, nucleotide material was monitored at 260 nm. Hexose was estimated as described by Chung and Nickerson [8] by means of the anthrone reaction, ribose by the orcinol reaction [9] and total phosphorus by a modification of the method described by Lowry et al. [10] using ATP as standard.

Solubilisation and lipid extraction of membranes

The membranes were suspended at protein concentrations ranging from 1 to 5 mg/ml in 50 mM Tris–HCl buffer (pH 7.5) containing 50 mM $MgCl_2$ or in water, as specified in Table I. The resulting suspensions were delipidated at 4 °C by extraction with *n*-butanol (0.4 ml of *n*-butanol per ml of membrane suspension [11]), either directly or after addition of 4 mg of dodecylsulphate per mg of protein. The resulting emulsion was centrifuged at $9000 \times g$ for 10 min. Three zones were obtained: an upper *n*-butanol phase containing most of the lipids, an insoluble interphase and a lower aqueous phase. The *n*-butanol phase was discarded, the interphase kept for analysis and the aqueous phase extracted again as before. Additional extractions with *n*-butanol did not cause any further difference in the amount of protein recovered as "soluble" or in the electrophoretic patterns (see below). In the following, the aqueous phase will be referred to as "soluble" fraction. The "soluble" character of all fractions was checked by centrifugation at $100\,000 \times g$ for 3 h. The fraction was dialyzed overnight at 4 °C against: 50 mM Tris (pH 7.5) or 50 mM Tris (pH 7.5) containing 0.1 % dodecylsulphate, the buffer used for dialysis depending upon the addition of dodecylsulphate, or not, to the membranes prior to *n*-butanol extraction. The non-diffusible fractions were analysed for their protein, phosphorus and carbohydrate contents.

In some experiments, after the membranes were solubilised with sodium dodecylsulphate as described above, they were treated with sodium dithionite (1.2 mg/mg protein) followed by iodoacetamide (1.2–2.4 mg/mg of protein) and then extracted with *n*-butanol as previously described yielding a first aqueous phase (S_1). In this case, the interphase was solubilised with the same buffer and extracted again to yield a new soluble fraction (I_s in Table I). I_i corresponded to the remaining insoluble material after this second extraction [12].

Polyacrylamide gel electrophoresis

Polyacrylamide gels (7 %) were prepared and the electrophoresis performed as described earlier [12] except that longer gels (11 cm) were used. Unless otherwise stated, the samples for electrophoresis contained 50–70 μg protein estimated by the method of Lowry et al. [10].

Proteins were stained with coomassie [13], carbohydrates were located with the HIO_4–Schiff base reagent [14]. Nucleic acid, acid mucopolysaccharides and other ionic polyelectrolytes were located with toluidine blue staining [15]. Lipid-containing materials were stained with sudan III by a modification of the method of Narayan and Kummeron [16] as described by Smithies [17]. After staining, the gels were scanned at 575 nm (for coomassie stain) and 560 nm (for HIO_4–Schiff) with a Gilford 2400 spectrophotometer equipped with the model 2410-S linear transport.

Treatment of the membrane with non-ionic detergents and chaotropic agents

The membrane suspensions were centrifuged at 27 000 × *g* for 20 min and the pellet resuspended at 2.5 mg protein/ml in a solution of the detergent or chaotropic agent and kept for 30 min at room temperature or incubated for the same period at 37 °C. After centrifugation as described above, the supernatants were collected and their protein contents and polyacrylamide gel electrophoresis patterns determined. The solutions in water unless otherwise stated, were as follows: 1 % Triton X-100; 1 % Nonidet P-42; EDTA, 5 or 30 mM; 30 mM EDTA in 0.2 M sodium acetate (pH 5.0) or in 50 mM Tris, pH 7.5; 1 % guanidinium · HCl, and 1 M NaCl.

Extraction of lipids from the "soluble" fraction

Samples of the "soluble" fraction (2 mg of protein each) were freeze-dried in glass centrifuge tubes, and the solids were extracted three times with 1-ml portions of ethanol–ether (3 : 2, v/v) at 4 or 25 °C. After centrifugation at low speed, the supernatants and sediments were recovered and their protein and phosphorus contents and acrylamide electrophoresis patterns determined. The gels were stained for proteins and lipoproteins (see above).

Separation of the components of the "soluble" fraction

"Soluble" fraction (3.6 mg of protein) in Tris–HCl buffer (50 mM, pH 7.5; 30 ml) whose phosphorus, hexose and ribose contents were known, was mixed with an equal volume of 20 % (w/v) trichloroacetic acid at 4 °C. After 20 min, it was centrifuged (27 000 × *g*, 20 min), the supernatant SN_1 recovered, and the sediment extracted at 25 °C with 0.1 % (v/v) $HClO_4$ in 70 % ethanol (30 ml), the supernatant SN_2 obtained by low speed centrifugation was kept and the pellet further extracted at 25 °C with 70 % ethanol (30 ml). A supernatant designated SN_3 was obtained in the same manner. The residue was extracted twice with chloroform–methanol (2 : 1, v/v) at 60 °C for 20 min as described by Folch-Pi et al. [18], yielding a fourth supernatant (SN_4). In other experiments the residue was extracted twice with 0.5 M $HClO_4$ at room temperature. The protein, phosphorus, hexose and ribose content of all supernatants was determined as described above.

Treatment with trypsin of the "soluble" fraction

The "soluble" fraction (1 mg/ml, 1 ml) was treated with 0.1 ml of trypsin solution (2 mg/ml) at 37 °C and after periods of 5, 10 and 20 min, samples (180 µl) were drawn. The reaction was stopped by the addition of 20 µl 10 % dodecylsulphate to each sample, and their gel electrophoresis patterns were compared with those of an untreated sample. The gels were stained for protein and glycoproteins as indicated above.

Gel filtration

Sephadex G-200 was equilibrated and eluted at room temperature with the buffers indicated in the legends of the figures. The void volumes (V_0) and the ($V_0 + V_i$) of each column were determined from the elution volumes of blue dextran (by absorbance at 650 nm) and L-tryptophan (absorbance at 280 nm), respectively. The flow rate was kept at 25–30 ml/h. Molecular weights of the different fractions were approximated according to the method of Determan and Michel [19].

TABLE I

YIELD OF SOLUBLE AND INSOLUBLE PROTEINS FROM *STREPTOMYCES ALBUS* G MEMBRANES BY *n*-BUTANOL EXTRACTION UNDER DIFFERENT CONDITIONS

For experimental details see Materials and Methods. As indicated in the text, sodium dodecylsulphate was added at 4 mg/mg membrane protein. Results are the mean values of different experiments (number between parentheses) ± standard error. Membranes were treated with RNAase (see Table II) prior to solubilisation with dodecylsulphate.

Membranes		Buffer	Percent protein recovery	
Pretreatment	Protein (mg/ml)		"Soluble"	Insoluble
None	1.3–2	50 mM Tris–HCl–5 mM $MgCl_2$	21.10 ± 4.2 (6)	25.41 ± 2.3
None	1.3	water	9.33	31.87
Dodecylsulphate	1.3–1.5	50 mM Tris–HCl–5 mM $MgCl_2$	19.01 ± 3.1 (5)	33.33 ± 5.8 (3)
Dodecylsulphate	1.5–2.5	50 mM Tris–HCl–5 mM $MgCl_2$	23.26 ± 3.2 (5)	48.38 ± 5.8 (3)
Dodecylsulphate	3–3.5	50 mM Tris–HCl–5 mM $MgCl_2$	25.37 ± 5.1 (11)	29.6 ± 4.0 (9)
Dodecylsulphate	4–5	50 mM Tris–HCl–5 mM $MgCl_2$	12.7 ± 1.0 (4)	42.6 ± 2.3 (4)
Dodecylsulphate + dithionite + iodoacetamide	1.1	50 mM Tris–HCl–5 mM $MgCl_2$	S_1* 35.08 I_s* 26.3	I_i* 3.77

* For definition of these fractions see Materials and Methods and ref. 12.

RESULTS

Effects of sodium dodecylsulphate and n-butanol on membranes. Preparation of "soluble" and insoluble fractions.

The results of *S. albus* membrane fractioning after *n*-butanol delipidation are shown in Table I. The protein recovery in the insoluble interphase varies with the membrane treatment prior to the extraction. Prior solubilisation of the membranes with dodecylsulphate increases it. However, the "soluble" fraction shows small variations except when membranes are suspended in water [20]. Its yield also depends upon the initial protein concentration with a maximum at 3 mg per ml. Nevertheless the protein recovery in soluble form is seriously affected by small changes in the temperature during *n*-butanol extraction. An increase of 2 °C produces an increment in the protein contents of the "soluble" fraction as well as in the number of bands of its electrophoretic pattern (see below). The protein recovered in "soluble" form increased when membranes were reduced and alkylated before extraction. However, this finding is not correlated with a modification of its electrophoretic pattern, as we shall see later on. It must be noted that the recovery of protein (soluble and insoluble) accounts for only 70 %. We have no obvious explanation for this loss of 30 % protein. It might be due to difficulties in estimation of protein in the insoluble fraction and/or to unaccounted partition of protein into the butanol phase.

Parallel work in our laboratory with *Micrococcus lysodeikticus* membranes [21] has shown the actual RNA content in the membranes is higher than that directly

measured with orcinol. We considered the measurement of the variation of ribose content with RNAase treatment and its subsequent partition after *n*-butanol extraction to be of interest. The results are illustrated in Table II. They show a reduction in RNA values after RNAase treatment, and a selective recovery of ribose in the soluble phase, as one could expect for nucleic acid material [22]. We should like to point out that the figures in the soluble fractions are even higher than those in the whole membranes. This confirms previous [12] findings. The phosphorus content behaves similarly.

TABLE II

RIBOSE (ORCINOL) AND PHOSPHORUS CONTENTS OF *STREPTOMYCES ALBUS* G MEMBRANES AND THEIR PARTITION AFTER *n*-BUTANOL MEMBRANE EXTRACTION

RNAase treatment was carried out in sodium acetate buffer as described under Materials and Methods. The amount of RNA was calculated by multiplying the ribose value by 5.2 [13].

Fraction	RNAase treatment	Ribose (mg/ml)	RNA (mg/ml)	Phosphorus (mg/ml)
Membrane	–	73.43	381.84	134
Membrane	+	18.18	94.54	n.d.
Soluble	–	183.58	954.62	277.5
Insoluble	–	<5	<25	52.6
Soluble	+	38.85	202.02	89.7
Insoluble	+	<5	<25	21.3

n.d., not determined.

Sodium dodecylsulphate gel electrophoresis of membranes and fractions

The dodecylsulphate electrophoretic patterns of the whole membrane and of its "soluble" and insoluble fractions are illustrated in Fig. 1. Gel a shows about 25 bands with two predominant groups of relative mobilities 0.84 ± 0.05 and 1.00 and a very faint staining of the minor (20–25) polypeptides groups. This seems to be a characteristic of these membranes. Furthermore, the stain of the minor components disappeared with aging of the membranes and/or with storage of the gels. Gel b corresponding to soluble fraction reveals a less complex protein pattern with three main groups of relative mobilities: 0.64 ± 0.05, 0.80 ± 0.05 and 1.0. They will be referred hereinafter as Groups 3, 2 and 1, respectively. Gels c and d show the pattern of the insoluble interphase at two different times after extraction. The pattern of Gel c is relatively similar to that of the "soluble" fraction but a change occurs with storage. Several points deserve further comment. The predominant group of relative mobility 0.84 ± 0.05 in the whole membrane is not found in these two fractions although a new heavily stained component (Group 3) is present in them. This may suggest a change in the electrophoretic mobility of the protein components of *S. albus* membranes by *n*-butanol extraction. This Group 3 stains more intensely in the soluble than in the insoluble fraction. Similar patterns were obtained from "soluble" fraction obtained from membrane non-solubilised with dodecylsulphate. The changes of the insoluble interphase with storage are striking. The protein bands of middle mobilities disappear and the zone of high mobility increases its staining. Proteolysis could easily explain this phenomenon. However, we have not been able to detect a clear proteolytic activi-

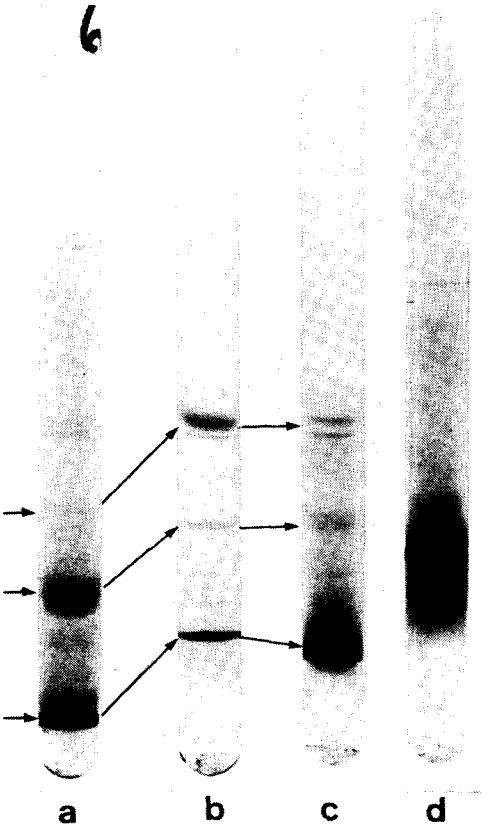

Fig. 1. Polyacrylamide gel electrophoresis in sodium dodecylsulphate of the whole membrane of *S. albus* and of its fractions obtained after *n*-butanol extraction. Conditions for extraction and electrophoresis are given in Materials and Methods. a, whole membrane; b, "soluble" fraction extracted from *S. albus* membranes (3 mg protein/ml) previously solubilised with dodecylsulphate (see Table I); c, insoluble fraction after 20 h extraction in the same conditions; d, insoluble fraction after 48 h extraction. Migration was towards the anode (bottom of the gels). The variations in migration reflects differences between experiments and samples. Note that the mobility of the fast-moving band corresponds in each case with that of the tracking dye (i.e. its relative mobility is 1.0). The arrows indicate the relative position of Groups 3, 2 and 1 from top to bottom.

ty in this fraction or in the whole membrane. Moreover, the presence of protease inhibitors (trasylol, soybean-trypsin inhibitor) did not protect the insoluble fraction from its change. Another possible explanation could be a modification in the state of aggregation of the proteins and thus of their hydrodynamic properties [23]. Preliminary experiments with more drastic dissociating treatments of the fraction (e.g. increase in dodecylsulphate concentration and/or time of boiling, presence of β-mercaptoethanol) partially reverse its change. Nevertheless, the reduction and alkylation of *S. albus* membranes prior to their *n*-butanol extraction did not essentially modify the electrophoretic patterns of the fractions (see Fig. 2), while increasing the amount of protein recovered as soluble (Gels a and b). In these conditions the pattern of the insoluble fraction (Gel c) was more stable on storage. It is worth noting that

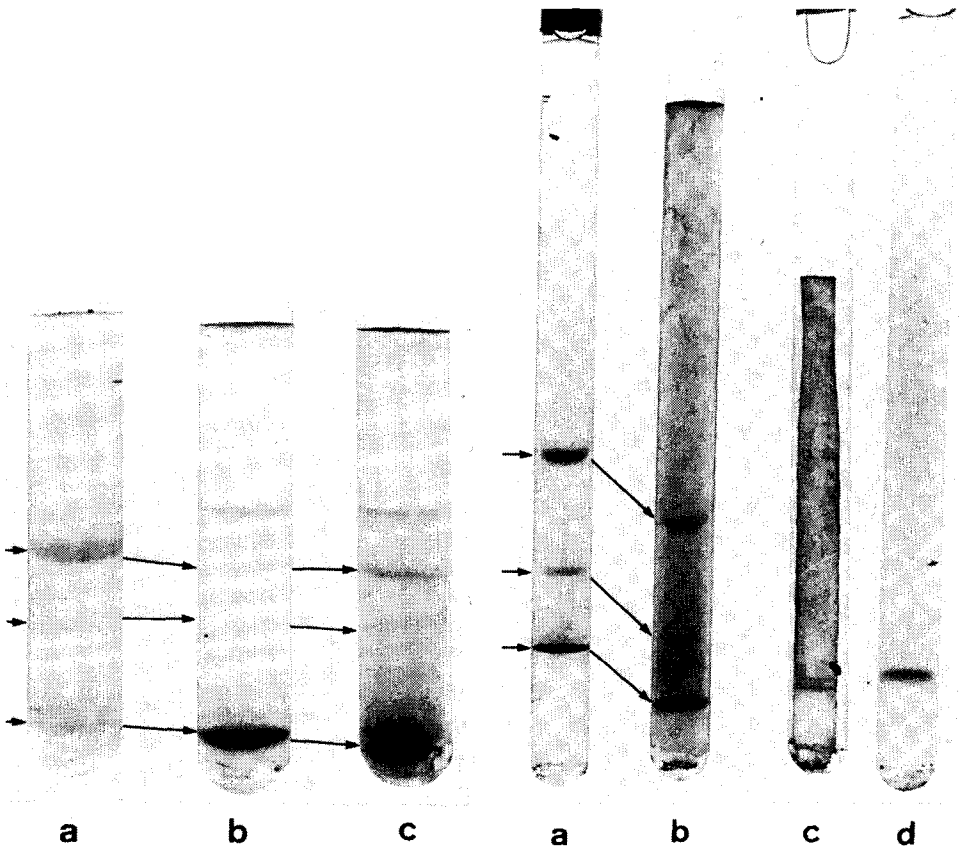

Fig. 2. Polyacrylamide gel electrophoresis in dodecylsulphate of the fractions obtained by *n*-butanol extraction of *S. albus* membranes previously treated with sodium dithionite and iodoacetamide (for experimental details see Materials and Methods and Table I). a, "soluble" fraction; b, "soluble" fraction from the first interphase solubilised and re-extracted; c, insoluble residue after second extraction. The arrows indicate the relative position of Groups 3, 2 and 1 from top to bottom.

Fig. 3. Specific stain of the components of the "soluble" fraction after polyacrylamide electrophoresis. "Soluble" fraction obtained from membranes previously solubilised with dodecylsulphate (for experimental details see the text). a, protein staining with coomassie brilliant blue; b, glycoprotein staining with HIO_4–Schiff reaction; c, staining with sudan III; d, nucleic acid and polyelectrolyte staining with toluidine blue. The arrows indicate the relative position of Groups 3, 2 and 1 from top to bottom.

the sum of the patterns of the two "soluble" fractions (Gels a and b) resembles that of the "soluble" fraction obtained from membranes without reduction and alkylation (see Gel b in Fig. 1). On the other hand, electrophoresis under non-dissociating conditions failed to give any pattern of protein.

Characterization of "soluble" fraction

Owing to the apparent complexity of the insoluble fraction, we have concentrated on the study of the components of the soluble fraction obtained after *n*-butanol extraction of non-reduced *S. albus* membranes. To further characterize them, we

TABLE III

FRACTIONATION OF THE DIFFERENT COMPONENTS OF *n*-BUTANOL SOLUBLE FRACTION FROM *STREPTOMYCES ALBUS* G MEMBRANES

Details of the extraction procedures and of protein, phosphorus, hexose and ribose determinations are given in Materials and Methods. Results are expressed as percentage of the initial amount of each component and are the mean values of duplicate experiments.

Treatment	Fraction	Protein	Phosphorus	Hexose	Ribose
Trichloroacetic acid (10 %, v/v)	SN_1	16.2	46.6	3	50.0
$HClO_4$–70 % ethanol (0.1 %, v/v)	SN_2	9.1	6.68	39.0	2.95
70 % ethanol	SN_3	0.64	9.15	4.7	0
Chloroform–methanol	SN_4	2.6	1.92	3	2.95
Ethanol–ether, 4 °C (2:1, v/v)	Supernatant	12	8.1	n.d.	n.d.
	Sediment	87.5	75.6	n.d.	n.d.
Ethanol–ether, 25 °C (2:1, v/v)	Supernatant	38.05	42.1	n.d.	n.d.
	Sediment	47	44	n.d.	n.d.

n.d., not determined.

analysed the possible presence in this fraction of carbohydrates, lipids and polyelectrolytes by specific staining procedures after dodecylsulphate electrophoresis. Results are given in Fig. 3. Gel b shows a positive HIO_4–Schiff reaction for protein groups 1, 2 and 3 (Gel a): Gels c and d reveal a component staining for lipid and a positive reaction for polyelectrolytes, respectively, both corresponding to Group 1.

To ascertain the complex nature of the proteins of this fraction, we examined the partition of its non-protein components in different solvents. The results are recorded in Table III. The high amounts of ribose and phosphorus remaining in the supernatant after precipitation with trichloroacetic acid indicate the presence of nucleotide material of low molecular weight, probably resulting from digestion of the fraction with RNAase (see above). About 45 % of the initial hexose is extracted with ethanol, therefore suggesting the existence of carbohydrate complexes in the fraction. Very small amounts of its components were subsequently extracted with chloroform–methanol. This can be taken as an indication of the absence of complex lipid material non-covalently bound to protein. Approx. 50 % of all components remained in the final residue after these extractions. It is worth to note that its further extraction with hot 0.5 M $HClO_4$ failed to extract more ribose. Electrophoretic analysis of all supernatants showed in SN_2 and SN_3 a sharp-fast-moving band (relative mobility 1.0), faintly stained for protein and strongly positive to the HIO_4–Schiff reaction. SN_1 revealed a very weak band of the same characteristics. SN_4 was free of protein and carbohydrate-staining material. Difficulties with regard to the total solubilisation of the final residue hampered its electrophoretic analysis. A partially solubilised material from this residue (25 % of its total protein) with 1 % dodecylsulphate at 37 °C for 1 h, showed a similar pattern for protein and carbohydrate stains to that of the original soluble fraction but with small increments in relative mobilities for Group 2 and 3.

Direct extraction of the *n*-butanol-soluble fraction with ethanol–ether, a procedure applied for delipidation of serum lipoproteins [24], released about 10 % of protein and phosphorus if extraction was performed at 4 °C and 40 % of both components at 25 °C; by electrophoresis the two supernatants showed only a fast band (relative mobility 1.0) which stains for lipid and protein, whereas the sediments gave the same patterns as the initial soluble fraction but with a weaker staining for lipid.

Apparent molecular weights of the polypeptide groups of "soluble" fraction

The apparent molecular weight of each group was calculated by plotting its relative mobility in the dodecylsulphate-polyacrylamide electrophoresis in a standard curve constructed according to Estrugo et al. [12]. The values obtained were 63 000±6 000 for Group 3, 40 000±4 000 for Group 2 and 17 000±2 000 for Group 1. Owing to the possible glycoprotein nature of most of these polypeptides, these results must be taken with caution [3].

Trypsin action on "soluble" fraction

Hence, it seems that the "soluble" fraction is very complex with non-protein components of low and relatively high molecular weights. Furthermore, its protein appears to be of glycoprotein nature. To confirm this, we examined the parallel sensitivity to trypsin of protein and carbohydrate staining using gel electrophoresis. Results are illustrated in Fig. 4 and show the complete disappearance of Group 3 and a marked reduction in Groups 2 and 1. The disappearance of these components was paralleled by an increase in diffuse material showing a positive Schiff's reaction beyond the tracking dye. More recently, we have been able to show that trypsin treatment of *S. albus* protoplasts transformed the Group 3 into components of faster mobility staining for protein and carbohydrate (Larraga, V. and Muñoz, E., unpublished). This suggests the glycoprotein nature of at least one protein group of this fraction and indicates that the other two groups may be constituted in part by carbohydrate-containing proteins.

Effects of non-ionic detergents, chaotropic agents and EDTA on membranes

In order to elucidate whether the solubilisation of these proteins after *n*-butanol extraction of *S. albus* membranes was dependent on their linkage to the membrane or simply to their glycoprotein nature [25], we studied the effect of different agents on the protein solubilisation of the membranes. The quantitative and electrophoretic results are presented in Table IV and Fig. 5, respectively. It should be pointed out that treatment of *S. albus* membranes with EDTA dissolved in water led to almost complete solubilisation. The effect of EDTA seems to be rather specific depending on the low ionic strength of the medium. The release of protein was actually diminished when EDTA solutions of the same pH in the presence of medium and high salt concentration were used. High NaCl concentration did not extract appreciable amounts of protein. On the other hand, guanidinium · HCl and non-ionic detergents solubilised the same amount of protein as did *n*-butanol extraction (see Table 1). Moreover, similar electrophoretic patterns were obtained for the fractions derived from membrane treatment with certain non-ionic detergents and EDTA in the presence of salt (see Fig. 5).

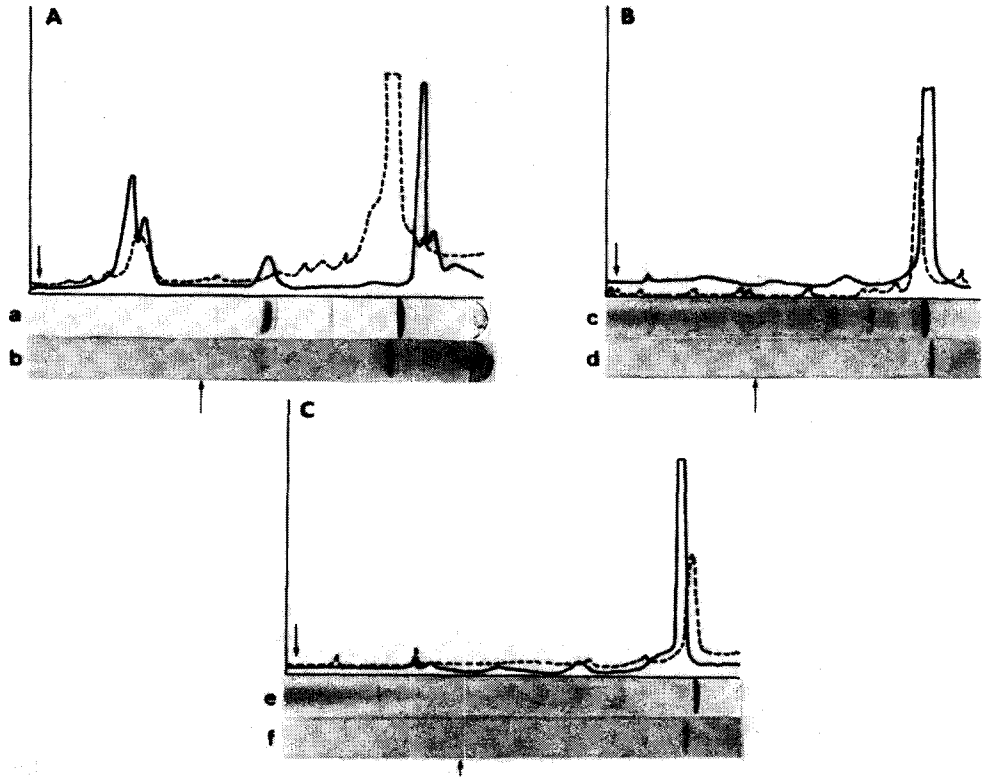

Fig. 4. Trypsin sensitivity of the "soluble" fraction components followed by protein and glycoprotein staining. (A) Control: a, protein (—); b, glycoprotein (- - -). (B) "Soluble" fraction after 5 min trypsin treatment: c, protein (—); d, glycoprotein (- - -). (C) Same fraction after 15 min trypsin treatment: e, protein (—), f, glycoprotein (- - -). Electrophoretic migration from left to right. The arrows indicate the origin of the tracings as compared with the gels. Different mobilities for a similar band simply reflect variations from run to run.

TABLE IV

PROTEIN SOLUBILISATION FROM *STREPTOMYCES ALBUS* G MEMBRANES BY TREATMENT WITH NON-IONIC DETERGENTS, CHAOTROPIC AGENTS AND SALINE SOLUTIONS

The membranes were extracted as indicated in Materials and Methods at the temperature specified in the table. Results are the means of at least duplicate experiments and are expressed in percentage of the initial protein.

Agent	Temperature (°C)	Protein
5 mM EDTA–water (pH 4.6)	25	98.1
30 mM EDTA–water (pH 4.6)	25	85.04
30 mM EDTA–0.2 M sodium acetate (pH 5.0)	37	17.7
30 mM EDTA–50 mM Tris–HCl (pH 7.5)	37	16.83
1 % Triton X-100–water	25	21.06
1 % Nonidet P-42–water	25	23.79
1 M NaCl–water	25	4.73
1 % Guanidinium · HCl	25	19.5

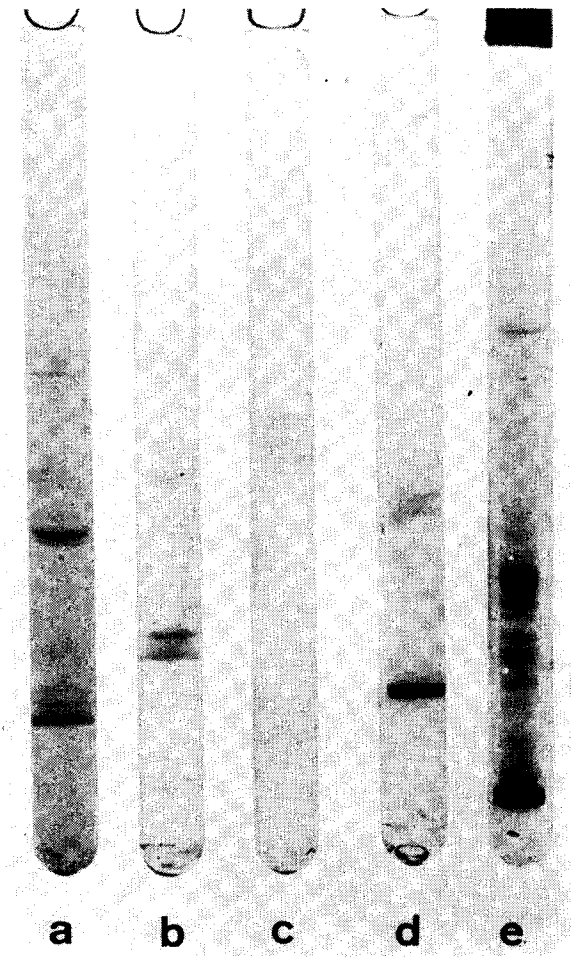

Fig. 5. Electrophoretic patterns of "soluble" fractions obtained by various treatments of *S. albus* membranes. a, 1 % Nonidet P-42; b, 1 % Triton X-100; c, 1 M NaCl; d, 5 mM EDTA in sodium acetate buffer (pH 5.0); e, 5 mM EDTA in water. The sames volumes of each fraction (200 μl) were deposited on the gels, the amount of protein depending upon the solubilisation efficiency. For experimental details see the text and Table IV.

State of aggregation of "soluble" fraction as examined by gel filtration

To study the "soluble" character of the fraction obtained by *n*-butanol extraction of dodecylsulphate-solubilised membranes, we examined its behaviour on Sephadex G-200. The fraction was exhaustively dialysed against NH_4HCO_3 buffer (pH 9.0) and eluted with the same buffer. Results are shown in Fig. 6. Three distinct peaks are observed, a first one eluted with the void volume (V_0) and the other two apparently richer in protein, eluted near the inclusion volume. These results tend to suggest that most of the protein components of the fractions were individualized and in a true "soluble" state. However, when the respective peaks were pooled and subjected to "standard" gel electrophoresis no protein band could be detected. If analysed by

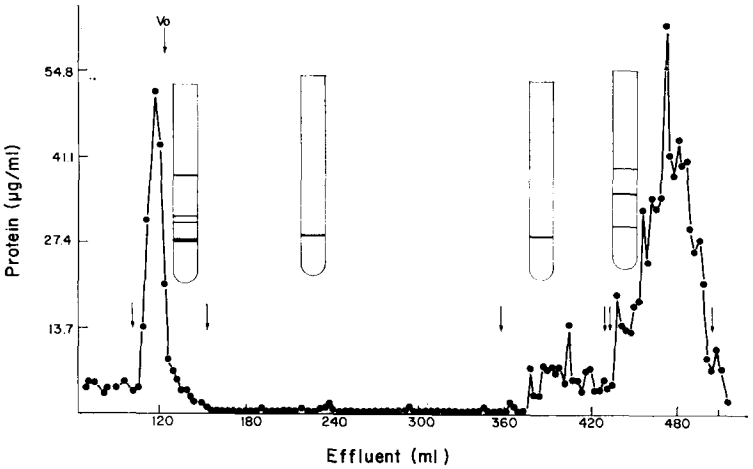

Fig. 6. Sephadex G-200 filtration of "soluble" fraction from *n*-butanol-extracted *S. albus* membranes. "Soluble" fraction from dodecylsulphate-solubilised membranes (20 mg Lowry protein/5 ml), exhaustively dialysed against 5 mM NH_4HCO_3 (pH 9.0), was charged on a column (2.5 cm × 80 cm) and eluted with the same buffer at room temperature. Protein was detected in the fractions by ultraviolet absorbance (280 nm) and the method of Lowry et al. [7]. The effluent was divided into four groups as indicated by the arrows; pooled and lyophilised gave rise to the F1, F2 and F3 and F4 fractions, respectively. After lyophilisation, the fractions were dialysed against several changes of the elution buffer. Schematic drawings of the dodecylsulphate-protein electrophoretic patterns of these fractions are shown.

the dodecylsulphate system, the first peak revealed a similar pattern to that of the soluble fraction, except for the presence of a double band in Group 2. This therefore suggests the existence of an aggregate. In fact, from the V_e/V_0 value of this peak we calculated a tentative molecular weight of 800 000 for it. Surprisingly enough, the other two peaks revealed in dodecylsulphate electrophoresis the same pattern as the whole fraction but weakly stained. This indicated a lack of correlation between the two procedures of protein detection.

Effect of sodium dodecylsulphate on the gel filtration of "soluble" fraction

In view of these results, we decided to examine the progressive dissociative effect of dodecylsulphate on its molecular sieving properties. First, the fraction was dialysed against 50 mM NH_4HCO_3 (pH 9.0) containing 0.1 % dodecylsulphate and subsequently dialysed against the same buffer without dodecylsulphate. The fractions were then chromatographed in the same column as above using 5 mM NH_4HCO_3 (pH 9.0) as elution buffer.

The results are shown in Fig. 7. The elution pattern is similar to that of Fig. 6, but the first peak appears now included in the column. A tentative molecular weight of 260 000 was assigned to it. Subsequent dodecylsulphate-gel electrophoresis of the first peak showed a complex pattern of protein corresponding to an aggregate with certain similarities to the "soluble" fraction. The peak F4 possessed an electrophoretic profile similar to that of F4 from Fig. 1. Fractions F2 (valley) and F3 (second peak) revealed by dodecylsulphate electrophoresis two bands of relative mobilities 0.8 and

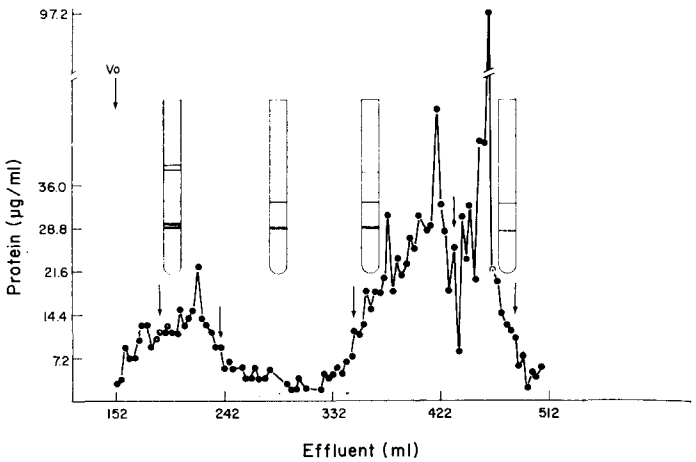

Fig. 7. Sephadex G-200 filtration of "soluble" fraction first dialysed against 5 mM NH_4HCO_3 (pH 9.0)–0.1 % dodecylsulphate and then dialysed against the same buffer without dodecylsulphate to remove the excess of detergent. The resulting sample (14 mg Lowry protein/5 ml) was charged on a column (2.5 cm × 80 cm) and eluted with 5 mM NH_4HCO_3 (pH 9.0) at room temperature. For other details see the legend of Fig. 1.

1.0. All these results confirm the existence of various states of aggregation for the "soluble" fraction as well as the anomalous detection of their protein content. At the same time, they suggest that these states may be dependent on the history of the protein (e.g. presence of dodecylsulphate during manipulations of the samples).

In subsequent experiments, the "soluble" fraction was added with 1 % dodecylsulphate and pretreated, or not, at 85 °C for 5 min and then fractionated on Sepha-

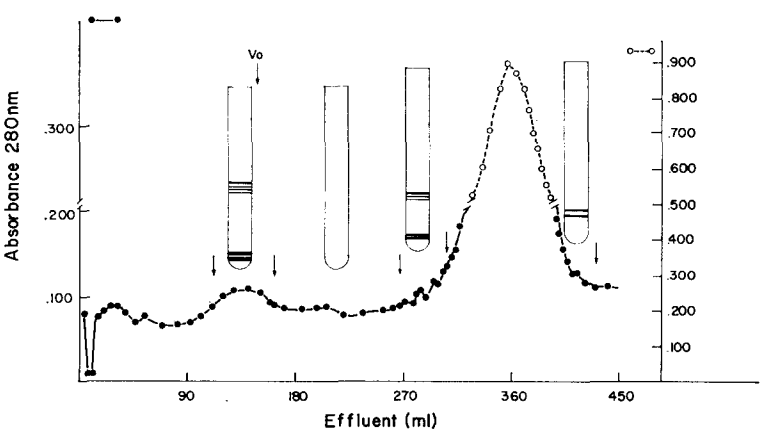

Fig. 8. Sephadex G-200 filtration of "soluble" fraction heated (85 °C, 5 min) with 1 % sodium dodecylsulphate and then dialysed against 50 mM NH_4HCO_3–0.1 % dodecylsulphate. The sample (14 mg Lowry protein/5 ml) was charged onto a column of the same conditions as above and eluted with 50 mM NH_4HCO_3–0.1 % dodecylsulphate at room temperature. Similar results were obtained with 50 mM Tris (pH 7.5)–0.1 % dodecylsulphate as elution buffer. The effluent was monitored at 280 nm (protein). For other details see the legend of Fig. 1.

dex G-200 with 0.1 % dodecylsulphate –50 mM NH_4HCO_3 (pH 9.0). The results are illustrated in Fig. 8. A marked change in the elution profile is observed. Only two distinct peaks are now detected: a small peak which elutes with the void volume and a second peak near the inclusion volume. Similar patterns were obtained for Fractions F1 and F3. These patterns and that of F4 differ from previous ones in a relative increase (as judged by the intensity of stain with the coomassie) in the components of faster mobility (Group 1) and the concomitant disappearance of components of Groups 3 and 2. No bands were detected in Fraction F2. The addition of β-mercaptoethanol did not essentially modify these results.

DISCUSSION

As far as we are concerned, this is the first report on the polypeptide profile of membranes isolated from an actinomycete. The importance of the Actinomycetales by their industrial applications and their use as possible models of cell differentiation is well known. By its complexity and the molecular weight of its components the protein pattern of *S. albus* membranes resembles those of other bacteria [4, 26]. However, the predominance of two polypeptide groups, as judged from their staining in dodecylsulphate electrophoresis is an emerging fact [4, 12, 27].

To characterize the proteins of the membrane, we first attempted their isolation after *n*-butanol extraction of the membrane. Similar procedures have been widely applied by Maddy [20] to the isolation of erythrocyte membrane proteins. Although no total protein solubilisation was obtained, we have succeeded in isolating a "soluble" fraction which is essentially formed by all, or part, of the two major polypeptide groups. Although more biosynthetic and structural work is needed, we have presented some evidence for the glycoprotein nature of most of these polypeptides. At this point, we should like to point out that part of the fast moving components seems to be a protein. We shall prove this in a future paper. There is increasing evidence that glycoproteins are constituents from bacterial membranes [28–30] and Baddiley, J. (communication at the First International Congress for Bacteriology).

The results obtained by gel filtration suggest that this fraction is formed by different aggregation states of its components. These states appear to be modified by the treatment of the isolated fraction. It is worth noting the vigorous action of dodecylsulphate (with or without β-mercaptoethanol) does not result in its complete dissociation. Furthermore, the properties of the protein components (i.e. detection, degree of dissociation and/or hydrodynamic characteristics) also vary with the treatment of the fraction and therefore with its states of aggregation. The simplest explanation for all these results taken together could be that the response to dodecylsulphate of the proteins isolated by us, differs from that of non-membrane proteins. A vigorous treatment with detergent may be needed for their complete dissociation. This would explain the modification of dodecylsulphate-gel electrophoresis patterns of the fraction from Fig. 8, leading to an apparent increase of lower molecular components and to the disappearance of Group 3. A similar situation has been observed for the major glycoprotein of erythrocyte membranes [31]. However, we should like to consider the alternative that these results may reflect changes in the hydrodynamic and/or conformational properties of the proteins. In an attempt to solve this question we have isolated the protein groups and studied their properties (see paper IV in this series).

There are an increasing number of examples showing changes in the state of aggregation and/or the conformation of membrane proteins depending on the experimental conditions, as well as on their history [32, 33]. The inadequacy of dodecyl sulphate and chaotropic agents in dissociating proteins and glycoproteins from brain and erythrocyte membranes has also been reported [31, 34–37].

Other interesting points are the complexity of the "soluble" fraction and the lack of resolution by electrophoresis under non-dissociating conditions. It must be noted that "soluble" fractions obtained from either dodecylsulphate-solubilised or unsolubilised membranes behave similarly this in respect. This indicated that this behaviour is not dependent on the state of the membrane during extraction and raised some questions about the true soluble character of the fraction.

Recent work by Rottem et al. [38] has presented evidence of heterogeneity for a soluble fraction from *Acholeplasma laidlawii* membranes extracted with *n*-butanol, as we previously showed for a solubilised interphase from *M. lysodeikticus* membranes [12]. The presence of lipids in such fractions may be of interest in the study of specific lipid–protein interactions, as well as the location of these complexes in the membrane. Butanol must essentially disrupt hydrophobic interactions. It is therefore difficult when it is used, to draw conclusions about the "extrinsic" nature [2] of the proteins recovered in the aqueous medium. However, the relatively similar results obtained by us with different types of solvents suggest that the proteins solubilised from *S. albus* membranes belong to a family of "extrinsic" proteins. A further study of their location is in progress.

The presence of RNA associated with *S. albus* membranes has been confirmed, We also have confirmed that RNA content is underestimated by direct application of the orcinol method to the membrane [30]. Its treatment with RNAase did not eliminate all the nucleotidic material. However, after the treatment, a great part of it appears to be of low molecular weight. This suggests that a specific interaction between nucleic acid and membrane components may exist. Although the possibility of an artifactual trapping cannot be ruled out.

Finally, we should like to point out that, except for their molecular weight, the gel profile and the solubility properties of *S. albus* membrane proteins show similarities with those of erythrocyte membranes [39].

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