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MEMBRANE RECONSTITUTION IN *chl-r* MUTANTS OF *ESCHERICHIA COLI* K 12

VI. MORPHOLOGICAL STUDY OF MEMBRANE ASSEMBLY DURING COMPLEMENTATION BETWEEN EXTRACTS OF *chl-r* MUTANTS

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SUMMARY

The particles formed during the complementation between supernatant extracts of mutants *chl* A^- and *chl* B^- of *Escherichia coli* K 12 have been examined under the electron microscope. These particles look like membranous formations with a vesicular structure limited by a triple-layered membrane. The observations made on these particles at various incubation times during complementation show that the aggregation progresses through several stages of organization. When the phenomenon becomes steady, the population of reaggregated particles shows a large structural heterogeneity: it consists of small aggregates, filaments and particulary well-organized closed vesicles. As we had previously shown for the reconstitution of nitrate reductase, the rate of formation and the structure of newly formed aggregates depend on various parameters (temperature, dialysis).

INTRODUCTION

The existence in the membrane of multimolecular entities containing phospholipids, proteins and divalent cations, has been suggested by Engelman and Morowitz[1] in order to explain membrane reaggregations in the presence of Mg^{2+} after solubilization by sodium dodecyl sulfate. Using detergents to disaggregate membranes implies that these compounds can break the bonds responsible for membrane cohesion, as stated by Green *et al.*[2].

This fact indicates the existence of lipoproteins giving, in the presence of detergents and particulary sodium dodecyl sulfate, clear solutions consisting of protein–sodium dodecyl sulfate complexes and lipid–sodium dodecyl sulfate micelles which analytical ultracentrifugation fails to separate from one another.

Engelman and Morowitz[1] have shown the actual existence of lipoproteins in solutions of membranes of *Mycoplasma laidlawii* treated by sodium dodecyl sulfate

Abbreviation: KDO = 2-keto-3-deoxyoctulosonate.

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and dialysed against a buffer containing no divalent cations. The molecular weight of lipoproteic particles, determined by the same authors is about 160000 ± 40000. Mg^{2+} is required to obtain reaggregation following orientations imposed by the membrane organization. Rottem *et al.*[3] and Razin *et al.*[4] have shown that dialysis against a buffer with a low Mg^{2+} concentration results in reaggregated membrane material consisting mostly of lipids, and when the Mg^{2+} concentration is increased, more protein is incorporated. Critical analysis of these results led Razin[5] to the conclusion that separated membrane components are able to interact spontaneously to form membrane-like material in the absence of a pre-existing membrane template. All these works deal with membrane reconstitution only from the morphological point of view and exclude the functional one. In contrast, Racker[6] and other workers have performed the reconstitution of the oxidative phosphorylation system and succinoxidase complex from mitochondrial membrane fractionated under mild conditions.

The synthesis of membranes in the cell consists, in all likelihood, of a sequence of events such as associations between phospholipids and proteins, the mechanism of which remains to be explained. As Salton[7] pointed out, the selection of mutants should be a good approach of this problem. From this point of view, the chlorate-resistant mutants of *Escherichia coli* K 12[8–14] go to make an ideal biological material. These mutants, selected by introduction of ClO_3^- into cultures of *E. coli* K12, wild-type strain, under anaerobic conditions, are defective for several enzymatic activities specific to anaerobic respiration. They have been classified into seven groups corresponding to the same number of genes: *chl* A^-, *chl* B^-, *chl* D^-, *chl* E^-, *chl* F^-, *chl* G^- and *chl* C^-. The first six are pleiotropic (phenotype *nit*$^-$ *gas*$^-$), whereas the last (*chl* C^-) has the *nit*$^-$ *gas*$^+$ phenotype. Azoulay *et al.*[15], Schnaitman[16] and Onodera *et al.*[17] have presented evidence showing that this mutation pattern is related to the membranous nature of the affected enzymatic activities.

The complementation, *i.e.*, the mixing of extracts of the two pleiotropic mutants *chl* A^- and *chl* B^- under well-defined conditions, results, on the one hand, in the restoration of membranous enzymatic activities lost in the mutants and, on the other, in the reconstitution of material sedimentable under ultracentrifugation[18]. In the course of this reaggregation phenomenon, proteins (some of them being enzymatic: reconstituted nitrate reductase and solubilized ATPase) and phospholipids incorporate into newly-formed particles[19,20].

MATERIALS AND METHODS

Organisms and growth conditions

E. coli K12, strain PA 601 (356 of our collection) chlorate-sensitive and its two mutants *chl* A^- (356_{15}) and *chl* B^- (356_{24}). These mutants, which were described previously[21,9], have the *nit*$^-$ *gas*$^-$ phenotype and are chlorate-resistant.

Cells are grown and harvested as previously described[8].

Preparation of cell-free extracts

Washed cells were suspended in 0.05 M Tris buffer (pH 7.6), disrupted in a French press under a pressure of 15000 lbs/inch^2, then centrifuged at 10000 rev./min for 15 min in a Sorvall RC2 B to remove cell debris. Crude extracts were then subjected to different procedures, depending on the study to be done.

(1) To obtain membranes for electron microscope examination or biochemical analysis, crude extracts were centrifuged at $82500 \times g$ for 30 min (Ti 50 rotor, Spinco Model L4). The pellets (particulate material) were suspended and washed in 0.05 M Tris buffer (pH 7.6) to obtain a suspension of heavy particles containing 7–15 mg protein per ml. The supernatant was centrifuged once again at $220000 \times g$ for 90 min and the new pellet was suspended in the same buffer to obtain the light particles (3–5 mg protein per ml).

(2) To perform complementation, the crude extracts were centrifuged twice at $220000 \times g$ for 90 min. The pellets were discarded. The supernatants, cleared of all sedimentable material, constituted the soluble fractions used to perform complementation studies.

Complementation

The supernatant extracts of the two mutants *chl* A^- and *chl* B^- were mixed under conditions described by Azoulay *et al.*[18]. The reaction system, containing equivalent amounts (10–15 mg protein per ml) of each extract, was incubated in a vacuum at 32 °C. The reaction can be stopped by sudden cooling and the introduction of air. The kinetics of particle formation were followed by nephelemetry at 600 nm as previously described by Riviere and Azoulay[22]. In order to separate soluble and particulate fractions, the complementation mixtures were centrifuged twice at $220000 \times g$ for 90 min after the reaction was over. The pellet of newly-formed particles was suspended in 0.05 M Tris buffer pH 7.6 (about 5 mg protein per ml).

Analysis of particulate fractions

On isopycnic gradient. Suspensions of native or newly-formed particles have been subjected to fractionation by the following procedure: 5 ml of suspension (1–5 mg protein per ml) were layered onto a sucrose isopycnic gradient (20–60%, w/v) then centrifuged at $100000 \times g$ for 15 h (SW 39 rotor, Spinco L4). The fractions were assayed for nitrate reductase activity and particle concentration.

By electrophoresis. 1 ml of particulate suspension (5–20 mg protein per ml) was subjected to zone electrophoresis on a sucrose gradient of 5–30% used as a stabilizer (Kepes, A. and Joseleau, D., personal communication). The electrophoretic column was filled with 50 ml sucrose gradient in 1 mM Tris buffer (pH 7.3). Electrophoretic migration was performed for 1 h under a tension of 400 V. 2-ml fractions were analysed as previously described.

Assay procedures

Nitrate reductase activity was measured by the rate of H_2 uptake, as described by Pichinoty *et al.*[23], in manometric systems containing H_2, hydrogenase, 9 mM benzyl viologen, 0.33 M phosphate buffer (pH 7), 0.25 M KNO_3 and enzyme extract. The specific activity is expressed as μmoles NO_3^- reduced/mg protein per h (units). Particle concentration was determined by nephelemetry at 350 nm. Protein concentration was determined by absorbance at 280 nm or according to the method of Lowry *et al.*[24]. RNA was determined as described by Moule[25]. KDO (2-keto-3-deoxy-octulosonate) was assayed according to the method of Weissbach and Hurwitz[26] modified by Cynkin and Ashwell[27] as reported by Colowick and Kaplan[28].

Preparation of specimens for electron microscopy

Thin sections. Membrane materials were prefixed in 0.1% OsO_4 in acetate–veronal buffer (pH 6.2) for 1 h, then fixed with 1% OsO_4 overnight at room temperature. This suspension was washed twice in the same buffer. The pellet was resuspended in 2% melted agar, then cut into small blocks of about 1 mm^3. These were treated for 2 h in a 0.5% uranylacetate solution according to Ryter and Kellenberger[29]. The blocks were then dehydrated through a graded ethanol series, then in propyleneoxide and embedded in Epon 812. All sections were cut with glass knives on a LKB Ultratome and poststained with lead citrate as described by Reynolds[30].

Negative staining. A small drop of the material to be studied was placed on a carbon-coated grid; the excess fluid was removed from the grid with a piece of filter paper. The grids were washed with few drops of 1% sodium phosphotungstate solution, pH 7.0, then dried briefly in air and examined at 80 kV with a double condenser and 50 μm objective aperture in a Siemens Elmiskop I electron microscope.

RESULTS

Location of nitrate reductase in E. coli K12 wild-type strain

According to Schnaitman[16] and Azoulay *et al.*[18], particulate nitrate reductase, specifically induced by nitrate under anaerobic conditions, can be regarded as a very good marker of bacterial membranes.

Membranous particles from wild-type strain 356 of *E. coli* K12 were prepared then subjected to fractionation on sucrose density gradient centrifugation as described in Materials and Methods. Fig. 1 shows the sedimentation pattern of heavy particles. These particles separate on the gradient to give 2 bands, the lightest of which has a buoyant density ranging from 1.10 to 1.17 and contains almost all nitrate reductase activity. Fig. 2 shows the sedimentation pattern of light particles: they exhibit one single peak, the buoyant density of which ranges from 1.10 to 1.20; this peak contains the whole of the nitrate reductase activity (300–360 units per mg protein).

These observations suggest that the particles with a buoyant density ranging from 1.10 to 1.20 are representative of bacterial membrane. Moreover, their KDO content, below $4 \cdot 10^{-9}$ molecules per mg protein, shows that they are little contaminated by fragments of lipopolysaccharides. However, they have an important contamination of RNA (0.25 mg per mg protein). Negatively stained preparations and thin sections of light particles show closed vesicles of diameter equal to 0.2 μm (Figs 3a and 3b). These vesicles, void of any cytoplasmic material, are limited by a membrane having all the features of unit-membrane profiles with an overall thickness of 60–80 Å and an average value of 75 Å.

Formation of particles by complementation

The complementation experiments have been performed as described in Materials and Methods. After 15 min of incubation, the hint of turbidity is seen in the reaction mixture; the intensity of this turbidity increases as a function of incubation time to a maximum at 120 min as shown by kinetic studies carried out by nephelemetry at 600 nm[22]. We have followed the appearance of sedimentable elements by examining samples, taken at various times, under the electron microscope. The following observations have been made. (a) During the first 2–3 min of incubation

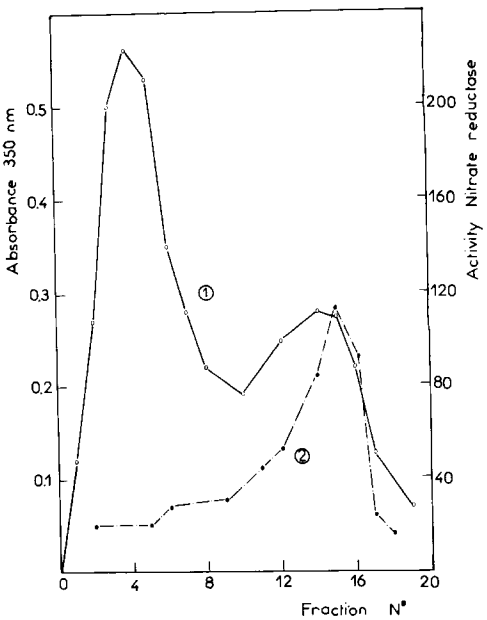

Fig. 1. Sucrose density sedimentation of heavy particles of *E. coli* K 12 strain 356 grown under anaerobic conditions in the presence of nitrate. 5 ml of suspension (1 mg protein/ml) were layered onto a sucrose isopycnic gradient of 20–60% (w/v), then centrifuged at $100000 \times g$ for 15 h. Curve 1, particle concentration expressed as absorbance at 350 nm. Curve 2, nitrate reductase activity expressed as μmoles NO_3^- reduced per h (units) per mg protein The top of the gradient is on the right.

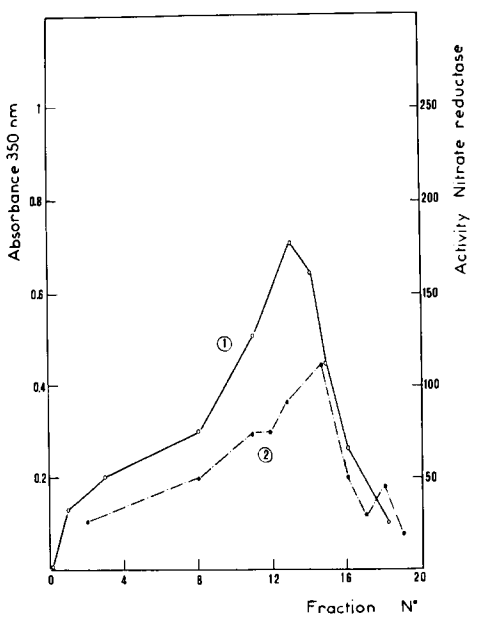

Fig. 2. Sucrose density sedimentation of light particles. Same experimental conditions as in Fig. 1. Curve 1, particle concentration expressed as absorbance at 350 nm. Curve 2, nitrate reductase activity expressed as units per mg protein. The top of the gradient is on the right.

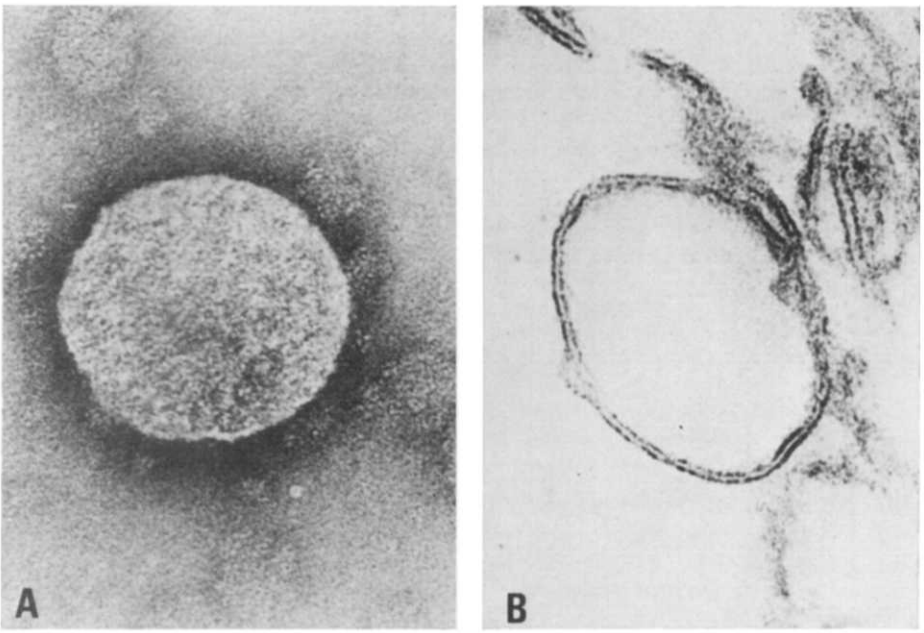

Fig. 3. Light particles, (A) Negative staining with phosphotungstate. (B) Thin section, uranyl acetate and lead citrate staining. Magnification: × 180000.

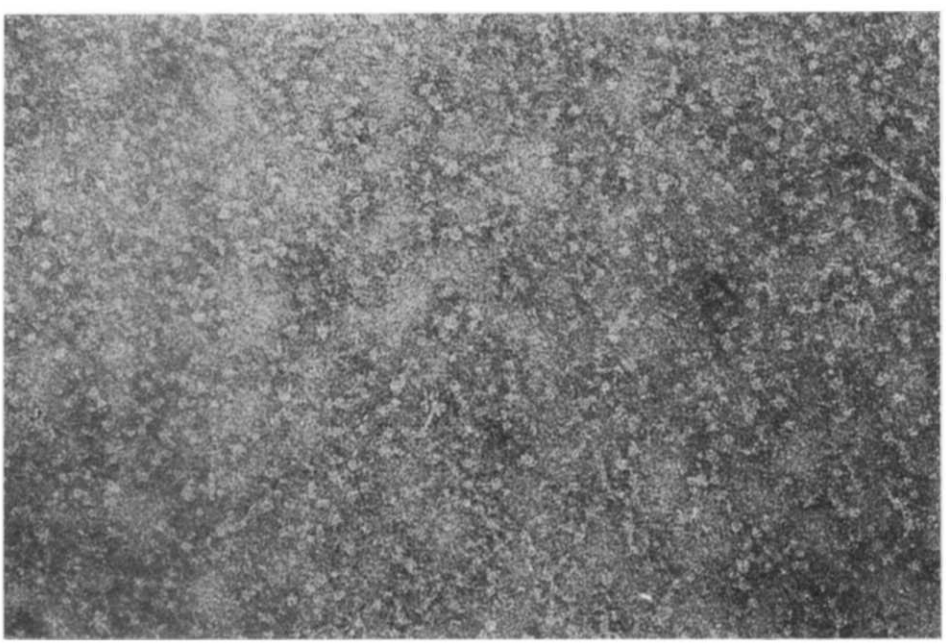

Fig. 4. Complementation: first 3 min of incubation. Negative staining with phosphotungstate. Only non-sedimentable aggregates are seen. Magnification: × 150000.

the reaction mixture shows the same pattern as controls (supernatant extracts of *chl A*$^-$ or *chl B*$^-$ strains): only small non-sedimentable aggregates are seen (Fig. 4). (b) After 15 min, a time necessary to obtain a pellet by ultracentrifugation, a beginning of organization is seen with small clearly-delineated regions of unit membrane (Fig. 5a). (c) After 90 min of incubation this organization becomes more precise: longer and more numerous fragments and some closed vesicles are seen (Fig. 5b). (d) After 120 min of incubation, when the phenomenon becomes steady and 10% of protein is passed from the soluble state into a particulate one, it is seen that aggregates have ceased to change and look like typical vesicles limited by a triple-layered membrane (Fig. 5c). It should be emphazised that the thickness of the membrane of these newly formed elements increases as a function of incubation time. At the beginning of the complementation, fragments are slightly less than 80 Å wide (Figs 5a and 5b) whereas at the end of the reaction some of them are 120–160 Å wide (Fig. 5c). Besides these characteristic vesicles, elements without well-defined structure are also seen.

These results imply that: (a) the complementation carried out by mixing supernatant extracts of mutant strains *chl A*$^-$ and *chl B*$^-$ leads to the reconstitution of particles, most of which look like membrane vesicles under the electron microscope. These newly-formed particles are responsible for the increase of turbidity during the complementation reaction; (b) this phenomenon progresses through successive stages, each of them resulting in a structure of higher organization; (c) when the complementation becomes steady, the population of reaggregated particles shows a large structural heterogeneity. It consists, on the one hand, of vesicles identical to native membrane vesicles and, on the other, of aggregates without well-defined organization. Razin *et al.*[4] have described a phenomenon analogous to ours in the reaggregation of solubilized membranes of *Mycoplasma laidlawii.*

Biochemical and morphological study of newly-formed particles

As previously shown, the complementation results in the formation of particles and the restoration of nitrate reductase activity lost by the two mutants *chl A*$^-$ and *chl B*$^-$.

The biochemical study of complementation mixtures has shown that 30–35% of nitrate reductase recovered at the end of the reaction was in a particulate state and that the specific activity of newly formed particles was equal to 20 units of nitrate reductase per mg protein. The examination of this material after the end of complementation was performed in negative staining in order to eliminate any artifact of fixation, dehydration and staining; it shows that the reaction mixture comprises a great variety of forms: vesicles of sizes ranging from 0.10 to 0.15 μm and filaments, probably made of proteins packed in a crystalline structure, the pictures of which look like those of Harris[31] in the case of proteins released from erythrocytes ghosts after sodium dodecyl sulfate treatment (Fig. 6).

Moreover, the newly-formed particles have been subjected to fractionation on sucrose density gradient. They separate on the gradient into three peaks of different buoyant densities (Fig. 7), but the major part of the newly-formed particles is concentrated in the two lightest peaks. The nitrate reductase activity is recovered in the lightest band, with a buoyant density ranging from 1.10 to 1.16. This result agrees with the observations made on nitrate reductase from particles of the wild-type strain. Samples of each peak have been examined under the electron microscope. In the

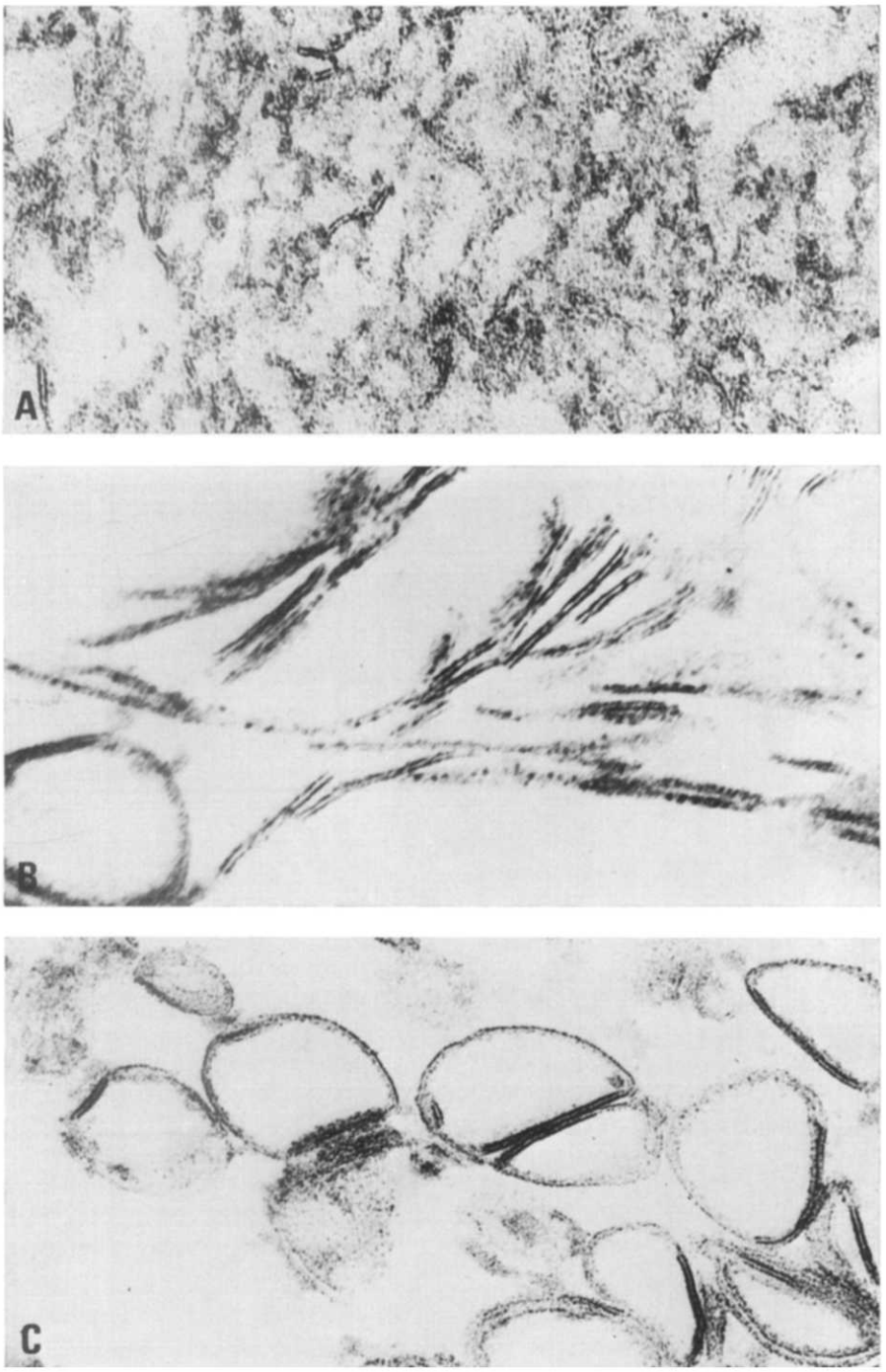

Fig. 5. Complementation. Thin sections of sedimentation pellets at various times of incubation. Uranyl acetate and lead citrate staining. Magnification: ×120000. (A) 15 min. A beginning of organization is seen: small clearly-delineated regions of unit membrane. (B) 90 min. The organization becomes more precise: longer and more numerous fragments and some closed vesicles. (C) 120 min. Characteristic membrane vesicles are seen.

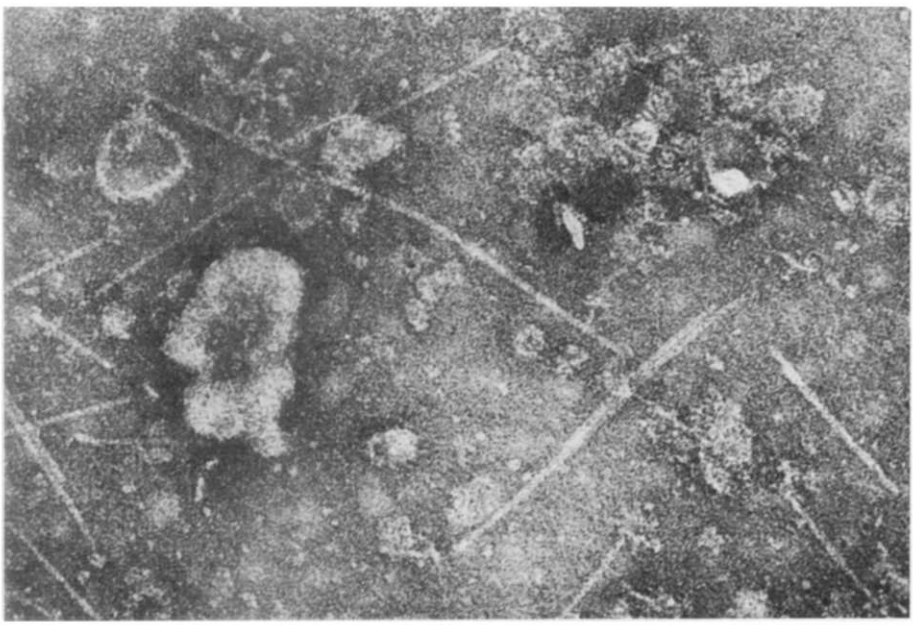

Fig. 6. Complementation particles. Negative staining with phosphotungstate. Very heterogeneous forms can be seen. Magnification: × 120000.

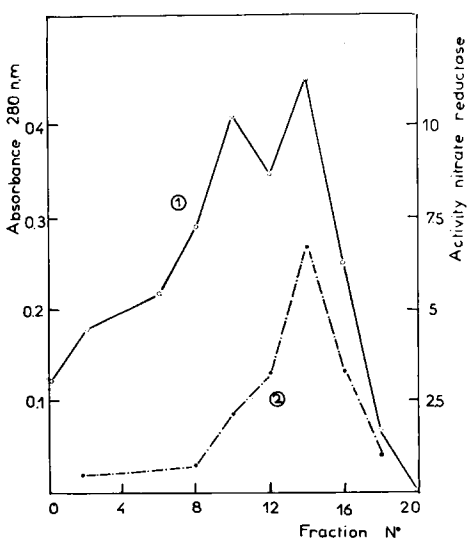

Fig. 7. Sucrose density sedimentation of particles formed during complementation after 120 min of incubation. 5 ml of suspension (1 mg protein/ml) were layered onto a sucrose isopycnic gradient of 20–60% (w/v), then centrifuged at 100000 × *g* for 15 h. Curve 1, protein concentration determined by absorbance at 280 nm. Curve 2, nitrate reductase activity expressed as units per mg protein.

lightest fraction, where reconstituted nitrate reductase is located (for this reason, this fraction might be considered as the most representative of a newly-formed membrane), few vesicles, short filaments and very small aggregates are seen (Fig. 8a). The second fraction (buoyant density about 1.18) is constituted of larger vesicles (Fig. 8b)

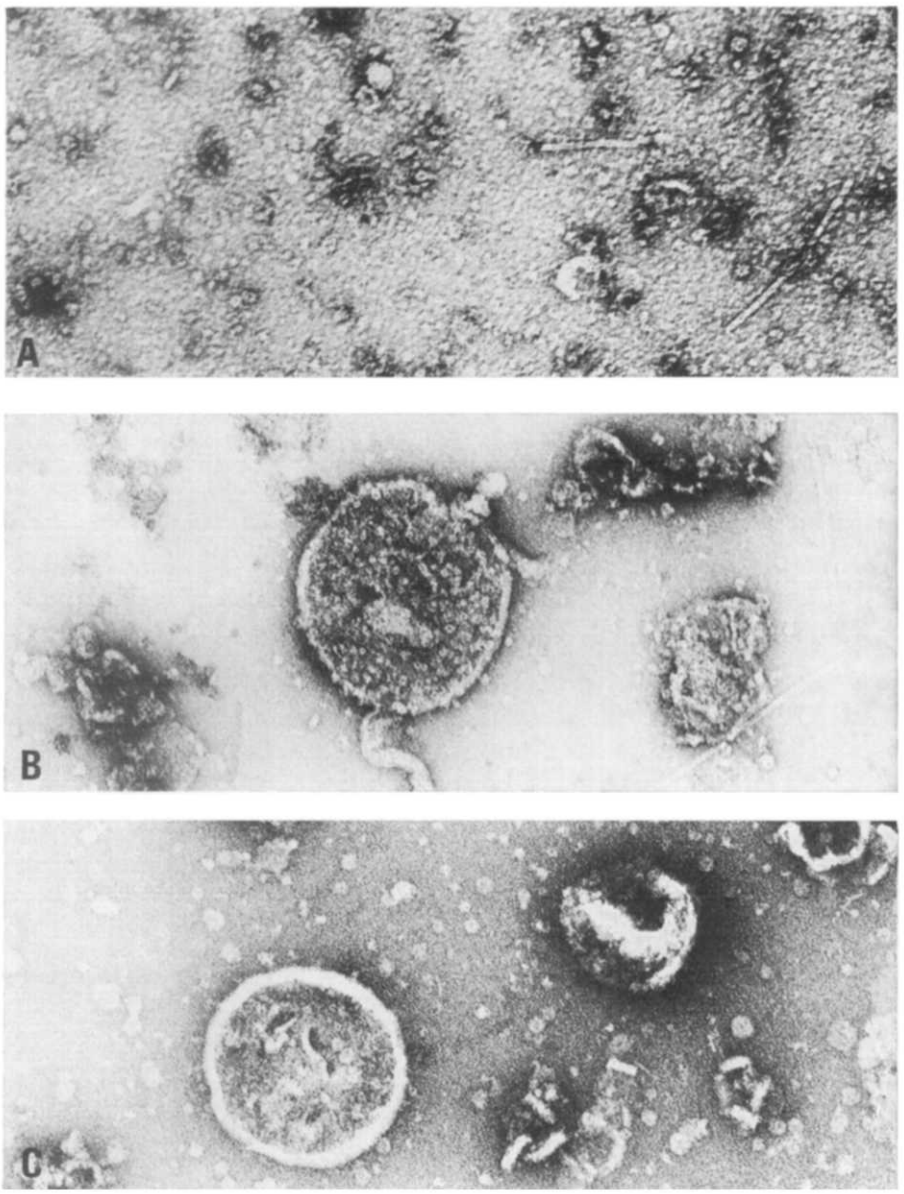

Fig. 8. Electron microscopy of fractions separated by sucrose density sedimentation. Negative staining with phosphotungstate. Magnification: × 120000. (A) Light fraction. Few vesicles, short filaments and very small aggregates are seen. (B) Middle fraction. Large vesicles, looking like native vesicles. (C) Heavy fraction. Characteristic vesicles.

looking like native vesicles. The third fraction, which is only a small proportion of the material sedimented after complementation (7% of protein content) contains characteristic vesicles and filaments identical with those described above (Fig. 8c). In this fraction, large and unvesiculated elements, similar to fragments released from the membranes of protoplasts by osmotic shock, are seen (Fig. 9).

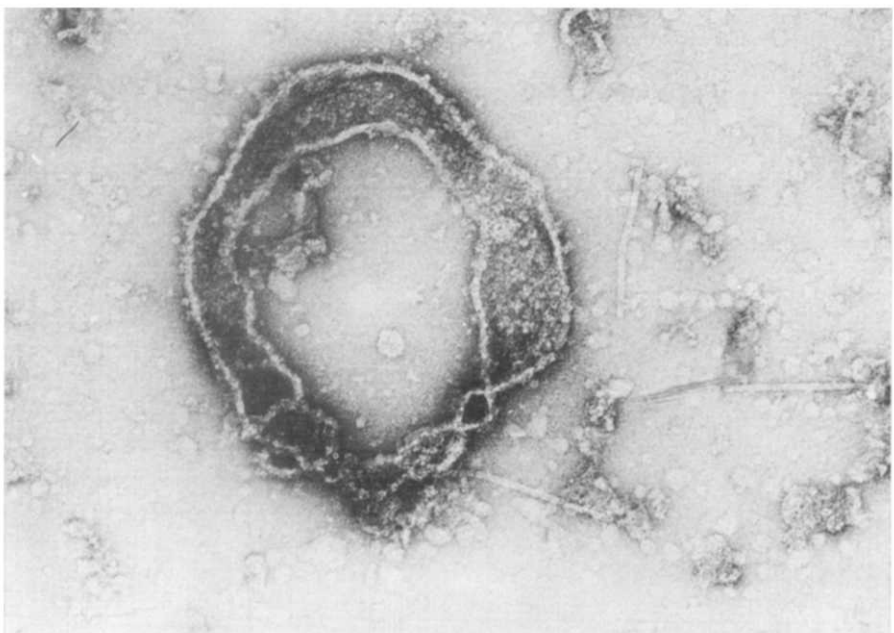

Fig. 9. Heavy fraction of newly-formed particles. Negative staining with phosphotungstate. Large unvesiculated sheets. Magnification: ×120000.

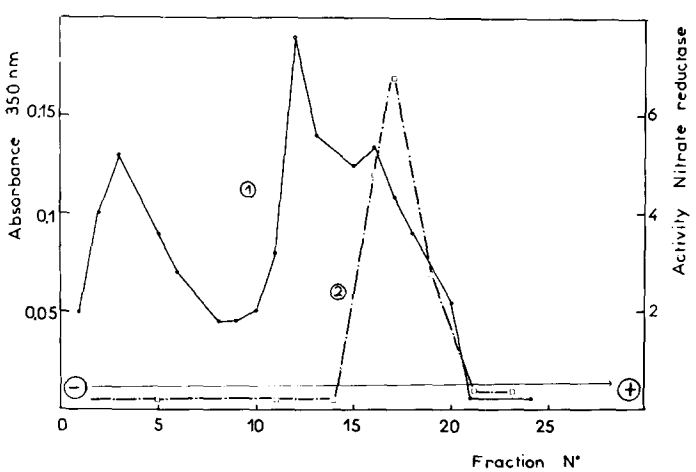

Fig. 10. Zone electrophoresis in a sucrose gradient used as a stabilizer. The electrophoretic column was filled with 50 ml sucrose gradient in 1 mM Tris buffer, pH 7.3. Electrophoretic migration was performed for 1 h under a tension of 400 V. Curve 1, absorbance at 350 nm. Curve 2, nitrate reductase activity expressed as units per mg protein.

Newly-formed particles have also been subjected to fractionation on zone electrophoresis as described in Materials and Methods. They separated into several peaks: nitrate reductase activity is concentrated in the band nearest to the anode (Fig. 10). It should be pointed out that these various fractions contain almost no KDO, which shows that they are little contaminated by fragments of cell wall.

Morphological study of particulate aggregates formed during complementation

Azoulay *et al.*[18] have shown that complementation is sensitive to temperature, and restoration of nitrate reductase activity is maximal at 32 °C. If the complementation is performed at 40 °C, the reaction mixture examined at the end of the reaction is devoid of any nitrate reductase activity.

Complementation experiments have been performed after dialysis of the supernatant extracts of *chl* A^- and *chl* B^- mutants against 0.05 M Tris buffer (pH 7.6) for 2 h in order to remove elements of small size and cofactors. Under such conditions the restoration of nitrate reductase activity is lowered by 60% and the particle formation begins only after 70 min of incubation at 32 °C. The addition of 0.1 ml of 10^{-5} M $MgCl_2$ to a 1-ml complementation system prepared with dialysed extracts results in a reactivation and leads to the formation of particles identical to those obtained under normal conditions. The addition of 0.1 ml 10^{-3} M NADPH to a 1-ml complementation system prepared with dialysed extracts results in a reactivation of the complementation bearing on the formation of particles which is achieved within 45 min whereas the rate of nitrate reductase restoration remains unchanged.

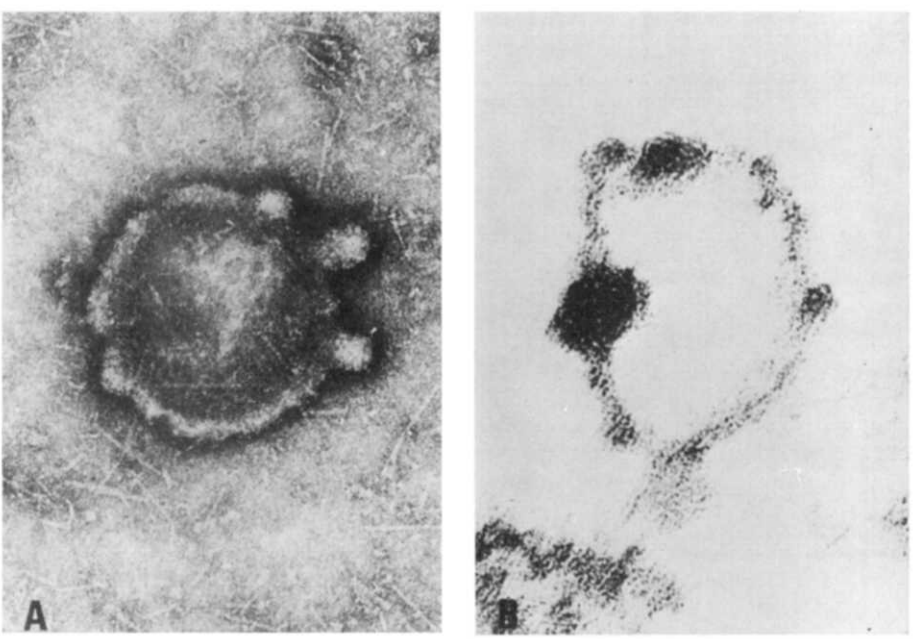

Fig. 11. Addition of 10^{-3} M NADPH to the complementation mixture of previously dialysed supernatant extracts. Vesicles, some of them showing buddings, are seen. (A) Negative staining with phosphotungstate. (B) Thin section, uranyl acetate and lead citrate staining. Magnification: × 120000.

The rate of particle formation reaches the value observed under normal conditions (without dialysis) and most of the particles obtained at the end of the reaction consist of vesicles, some of them showing buddings (Figs 11a and 11b). Our pictures look like those of Engelman and Morowitz[32] in the case of reaggregation of solubilized membrane by dialysis against Mg^{2+}-containing buffer. The addition of NADPH to a normal complementation mixture (without previous dialysis) results in the formation of twisted sheets consisting of granular aggregates (Fig. 12).

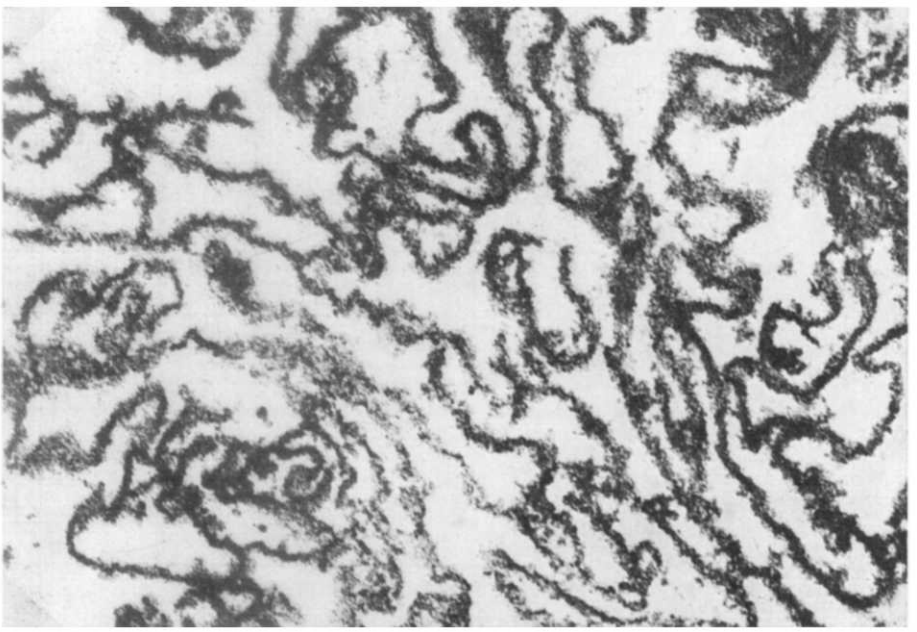

Fig. 12. Addition of 10^{-3} M NADPH to a normal complementation mixture (without previous dialysis). Long filaments constituted of granular aggregates are seen. Thin section, uranyl acetate and lead citrate staining. Magnification: ×40000.

DISCUSSION

We had previously advanced the hypothesis of an alteration of the cytoplasmic membrane in order to explain the pleiotropic effect of chlorate-resistant mutations in *E. coli* K12[13]. This assumption was founded on the restoration of nitrate reductase, an enzyme specifically bound to membrane, by complementation between the supernatant extracts of *chl* A^- and *chl* B^- mutants[15]. We had observed that: (a) nitrate reductase is reconstituted as two forms, a soluble one and a particulate one[18]; (b) the particulate form has properties similar to those of native membranous enzyme from wild-type strain[33]. During complementation, the restoration of nitrate reductase activity is attended by the formation of turbidity. When the reaction is over, 10% of soluble proteins are incorporated into particles which can be sedimented by ultracentrifugation.

The observations reported in this paper, particularly dealing with the morphological study of sedimentable elements formed during complementation, show that these elements have a structure which allows them to be identified as membrane

components. These results agree with those of Azoulay[20] regarding protein and phospholipid composition. In the same paper we had shown the part played by membrane lipids in the reconstitution of particles: the extracts of cells grown on a ^{32}P medium contain phospholipids and/or lipoproteins in a non-sedimentable form; when the complementation is over, 90% of phospholipidic ^{32}P is seen in the newly-formed particles. In another paper[33] we had shown that the treatment of the soluble fractions with phospholipase C before incubating results in a complete inhibition of the reconstitution of particles. Recently, preliminary work shows that the soluble extracts of the *chl-r* mutants contain lipoproteic fractions labelled by [2-^{3}H]glycerol which can be separated by Sephadex G-200 filtration and are able to be incorporated into the newly-formed particles.

Moreover, our results do not agree with the observations made on the reconstitution of membranes after solubilization in *Mycoplasma laidlawii* by Razin *et al.*[4], Engelman and Morowitz[1], Terry *et al.*[34] by the fact that in the systems of these workers, reconstitution depends on the presence of Mg^{2+}, whereas in ours it is the mixing of two cell-free extracts containing, probably, membrane components in a soluble and inactive state which leads to this reconstitution. During membrane solubilization by certain detergents, some of the morphopoïetic factors of membrane synthesis could be denatured. The absence of these factors could lead to the reconstitution of structures identical to membranes from the morphological point of view but deprived of enzymatic activity. Our results agree with those of Yamashita and Racker[35] who have succeeded in reconstituting the succinoxidase complex of mitochondrial membrane from its individual components, succinate dehydrogenase, cytochromes *b*, *c* and c_1, cytochrome oxidase, phospholipids and ubiquinone, mixed and incubated at 37 °C for several hours.

The examination of the aggregates formed during the complementation phenomenon under the electron microscope shows a large heterogeneity: the population of newly-formed particles comprises well-organized closed vesicles, filaments and small aggregates. From the biochemical point of view, the reconstitution of particles is attended by a restoration of nitrate reductase activity, 30–35% of which is incorporated into newly-formed particles. These particles separate on sucrose density gradients to give three peaks; the lightest of the particles contain the whole of the enzymatic activity, and are heterogeneous from the morphological point of view.

At this moment we are not able to state precisely whether reconstituted nitrate reductase is present in each particle of the light fraction no matter what may be its level of organization. If we assume that the restoration of nitrate reductase activity during complementation does not start before a given level of organization is attained, we cannot yet state precisely whether this particulate fraction contains an inactive polymer at various organizational levels. We hope that, from our future work, we will obtain the information concerning these two points which will enable us to understand better the assembly mechanism.

The patterns of the kinetics of the reconstitution[22] probably agree with a sequence of increasingly complex organization stages: aggregates, non-closed trilaminar structures, closed membrane vesicles identical to native vesicles. Nevertheless, it should be pointed out that these various stages are not fully homogeneous. It may be noted that the reconstitution phenomenon is attended by the binding of ATPase, which is solubilized during the extraction procedure, to the newly-formed particles,

as described by Giordano *et al.*[19]. After fractionation of these particles on a sucrose density gradient, ATPase can be recovered in two peaks of different buoyant densities. These results suggest that the heterogeneity of particulate forms observed during complementation is due to the fact that some of the stages of the organization process are not achieved. This assumption can be better appreciated when our results are compared with those obtained in the case of membrane reaggregations after solubilization by sodium dodecyl sulfate. In our model, the concentrations of the various components taking part in reconstitution are determined at the starting point, and the addition of a specific proteic factor (product of the gene *chl* B^+) to supernatant extracts, which do not contain it in an active form, results in the initiation of the process. In the case of the systems of Engelman and Razin, the presence of Mg^{2+} and the dialysis permitting the removal of sodium dodecyl sulfate promote reaggregations leading to structures similar to those obtained by us during complementation. In our case we had observed that dialysis of our supernatant extracts against Tris buffer results in a 50% inhibition of complementation; this inhibition is suppressed by the addition of Mg^{2+} at low concentrations[20] or of NADPH. The influence of the later compound is much more relevant when resulting aggregates are examined under the electron microscope. At the moment, however, we cannot explain the part played by this coenzyme.

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