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CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*

III. THE FORMATION AND AGGREGATION OF SMALL LIPOPROTEIN STRUCTURES DERIVED FROM SODIUM DODECYL SULFATE-SOLUBILIZED MEMBRANE COMPONENTS*

DONALD M. ENGELMAN** AND HAROLD J. MOROWITZ

Department of Molecular Biophysics, Yale University, New Haven, Conn. (U.S.A.)

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SUMMARY

Small lipoprotein aggregates are formed when sodium dodecyl sulfate-solubilized membrane components are dialyzed against buffer in the absence of divalent cations. These aggregates sediment as a single peak in analytical ultracentrifugation, and their behavior on sucrose density gradient centrifugation indicates that the lipid is bound to the protein. Upon dialysis against buffer containing 0.02 M Mg^{2+}, the small pieces further aggregate to yield structures having a membrane-like appearance in the electron microscope. The collection of larger aggregates has been designated M_r' (re-aggregated membrane prime) and has a buoyant density of 1.18 ± 0.05 g/cm^3. M_r' is similar to the original membrane in having the same protein to lipid ratio and the same magnesium to protein ratio.

INTRODUCTION

In earlier work[1] it was reported that the detergent sodium dodecyl sulfate solubilizes membranes isolated from *Mycoplasma laidlawii*, strain B, and produces separate lipid–detergent and protein–detergent complexes. It was observed[2] that removal of the detergent by dialysis against Mg^{2+}-containing buffer can induce the formation of membranous aggregates similar to the original membranes. Recombination of the protein and lipid occurs when Mg^{2+} is omitted, but only small aggregates are formed. The nature of the small lipoprotein aggregates and their interaction in the presence of Mg^{2+} constitute the subject of this paper.

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** Present address: Cardiovascular Research Institute, University of California Medical Center, San Francisco, Calif., U.S.A.

MATERIALS AND METHODS

Membrane isolation

Growth of *M. laidlawii* B, introduction of [^{14}C]lipid label, and procedures for the isolation of purified membranes have been previously described[1].

Solubilization and aggregation procedures

In order to avoid radical alteration of the membrane components[2], sodium dodecyl sulfate at a concentration of 10 mM was used to solubilize a suspension of purified membranes in a 1:20 dilution of β-buffer (β-buffer is: NaCl, 0.156 M; Tris, 0.05 M; 2-mercaptoethanol, 0.01 M; in deionized water, adjusted to pH 7.4 with HCl). The membrane was at a concentration of approx. 3 mg protein per ml. The solubilized material was then centrifuged at 48000 $\times$ g for 30 min and the very small pellet discarded. The supernatant was dialyzed for 36 h against 1:20 β-buffer with changes of buffer every 12 h. No turbidity increase similar to that seen in dialysis against Mg^{2+}-containing buffer was seen and centrifugation for 30 min at 48000 $\times$ g failed to sediment any appreciable amount of material.

An analysis of the removal of detergent was conducted using ^{35}S-labeled sodium dodecyl sulfate (New England Nuclear Corp.) to produce the initial solubilization of the material. Each of the changes of dialysis buffer was analyzed for radioactivity and the final dialysate was also analyzed. Progressive release of detergent was observed; the final dialyzed material contained only 1.7 % of the initial counts. Since the material failed to sediment on centrifugation for 30 min at 48000 $\times$ g, no estimation of the proportion of residual detergent bound to membrane components was possible.

To investigate the aggregative properties of the dialyzed material in the presence of Mg^{2+}, a solution of dialyzed material was dialyzed against 1:20 β-buffer containing 20 mM Mg^{2+}. After 48 h of dialysis a slight increase of the turbidity of the solution appeared, although the increase was small by comparison with that observed in the production of reaggregated membrane[2]. The dialysis was continued for 36 h against three changes of 1:20 β-buffer without Mg^{2+} in order to eliminate any aggregation of a non-specific character and to remove excess Mg^{2+}. The turbidity was retained after the second dialysis.

Density gradient centrifugation

Linear 4.6 ml 20–45 % (w/w) sucrose density gradients were formed using sucrose solutions made up in 1:20 β-buffer. Samples of the dialysate without Mg^{2+} were layered on the gradients and centrifuged at 40000 rev./min for 30 h at 25° in an SW 50 rotor and Spinco Model L-2 ultracentrifuge, 10 drop gradient fractions were bottom-collected, diluted with deionized water, and analyzed for protein and radioactivity.

Analytical ultracentrifugation

Samples of the material dialyzed without Mg^{2+} were placed in 4° sector cells and centrifuged at 50740 rev./min in a Spinco Model E analytical ultracentrifuge at 20° and examined using Schlieren optics. Values of $s_{20,w}$ were obtained from micro-comparator measurements of the photographic plates, and the tolerances given reflect maximal variation of the fit of the experimental points with a straight line.

Electron microscopy

The procedures used have been described previously[2]. In summary, material was fixed with formaldehyde, post-fixed with osmium, stained with uranyl acetate, embedded in araldite, sectioned and post-stained with lead citrate. The sections were examined using a Philips EM 200 electron microscope.

Assay procedures

Protein was determined using the Folin phenol method of LOWRY *et al.*[3] with egg white lysozyme as standard (2 × crystallized, Worthington Biochemical Corp.). Radioactive samples were dried on planchets and counted on a Beckman low-beta thin-window proportional counter.

Mg^{2+} was determined using a Perkin–Elmer model 303 atomic absorption spectrophotometer. Concentrations were read from calibration curves run concurrently with the samples.

RESULTS

Formation of small lipoprotein aggregates

Previous experiments[1] have shown that the protein and lipid of the membrane appear to be separated from each other as a result of detergent solubilization. Almost all of the solubilized membrane material remains soluble (*i.e.* fails to sediment at 48000 × *g*) when the detergent is removed by dialysis in a low ionic strength, Mg^{2+}-free environment.

During dialysis in the absence of Mg^{2+} a small but variable fraction of the lipid diffused out of the dialysis bag (up to 11 % of the total lipid). In the presence of Mg^{2+} less than 0.3 % of the lipid entered the dialysis buffer. The loss of lipid upon dialysis in the absence of Mg^{2+} may result from the relative instability of micellar aggregates in a low ionic strength environment.

In order to assess the state of the dialyzed material, sedimentation in sucrose gradients was employed. The dialyzed material was layered onto 20–45 % sucrose gradients in sample volumes of 0.4 ml. After centrifugation for 30 h at 25° and 40000 rev./min in the Spinco SW 50 rotor, fractions were collected and analyzed for lipid and protein content. A typical profile is shown in Fig. 1. The majority of the lipid and protein present is found in a single peak which has moved approx. 30 % of the way down the gradient. The lipid and protein curves have almost identical shape across the peak, so the protein to lipid ratio is roughly constant. A small amount of protein is seen trailing the peak and is not apparently accompanied by any lipid. The observation of such behavior has been made in a number of experiments and seems to be a consistent property of the dialyzed material.

As there is little detergent present in the preparation, it is to be expected that the free lipid molecules or micellar aggregates would have a buoyant density of no greater than that of crystalline phospholipid or 1.05 g/cm³ (ref. 4). Since the specific gravity of the 20 % sucrose at the top of the gradient is 1.08, the lipid components would not be expected to sediment if they are not bound to material of higher buoyant density, and it follows that the lipid observed in the sedimenting peak must be bound to protein. The constancy of the lipid to protein ratio across the peak suggests that the binding is reasonably stoichiometric, with the reservation that not all of the

protein present is bound. If a stoichiometric complex is being observed, it is possible that the unbound protein results from depletion of the phospholipid during dialysis resulting in insufficient lipid for binding to the protein.

Failure of the complex to sediment under conditions used to sediment membrane fragments may be taken as evidence of small particle size. The lack of turbidity in the dialysate is support for this view. Comparison of Fig. 1 with the sedimentation of the components in the presence of sodium dodecyl sulfate shows that the material in the absence of sodium dodecyl sulfate has migrated 30 % of the distance down the gradient while the protein peaks shown at various sodium dodecyl sulfate concentrations (under the same conditions of centrifugation) have migrated only 20 % of the distance (see ref. 1, Fig. 7, d–f). The lipid–protein complex may therefore be considered to be larger than the protein–sodium dodecyl sulfate complex seen in detergent solution since its buoyant density is probably lower.

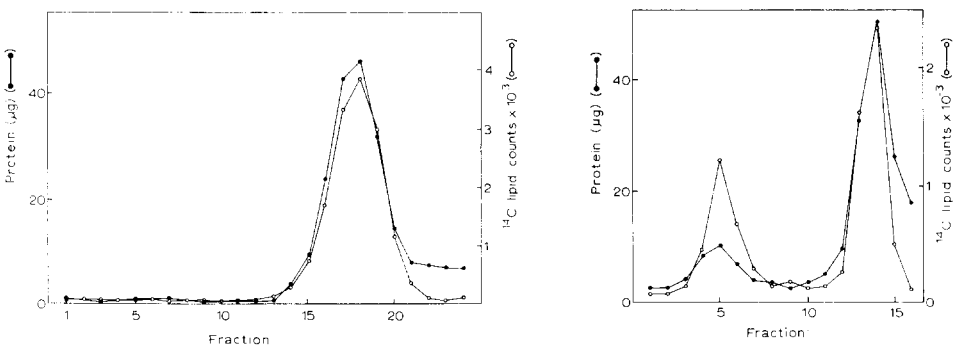

Fig. 1. Sucrose density gradient ultracentrifugation of small lipoprotein aggregates. Sedimentation carried out 30 h at 40000 rev./min on a 20–45% sucrose gradient. Curves for protein (●—●) and lipid (○—○) are shown.

Fig. 2. Sucrose density gradient ultracentrifugation of a mixture of original membrane and small lipoprotein aggregates. Sedimentation carried out for 8 h at 35000 rev./min on a 25–50 % sucrose gradient. Curves for protein (●—●) and lipid (○—○) are shown.

To demonstrate that the dialyzed complex is distinct in behavior from the original membrane and does not interact significantly with it, a mixture of membranes and dialyzed material was layered onto a 25–50 % sucrose gradient and centrifuged at 35000 rev./min for 8 h. The analysis of fractions for protein and lipid content is seen in Fig. 2. The original membrane preparation is presumed to have moved to a position of equilibrium banding and the dialyzed material is not seen to interact significantly with the original membrane.

The analytical ultracentrifuge was employed to determine the size of the lipoprotein aggregates seen in the material dialyzed without Mg^{2+}. The pitfalls which plagued such an analysis of the solubilized material should not apply in this case, since very little sodium dodecyl sulfate is present and since the system appears to contain primarily one type of component, *i.e.* lipoprotein aggregates. Two kinds of information can be derived from the sedimentation pattern. First, the character of the peak or peaks seen can give an idea of the homogeneity of the system. Second, an approximate measure of the size of the sedimenting particles can be derived. Four preparations of dialyzed material from three purified membrane isolates were examined. The pattern observed is shown in Fig. 3.

A single peak is seen which is skewed toward the trailing edge. The sedimentation coefficients ($s_{20,w}$) from separate experiments are 4.8 ± 0.2, 5.4 ± 0.4, and 4.4 ± 0.5 S. The variation in sedimentation coefficient is considerable, but may not exceed the confidence limits. A small population of larger particles is thought to be represented by the small irregularities in the schlieren line which precedes the main peak. The sedimentation coefficients are seen to be considerably higher than those observed for the solubilized material (2.9–3.58)[5], which is in agreement with the observation made of the relative sedimentation of the materials in sucrose gradients.

In order to obtain an estimate of the size of the small lipoprotein aggregate and some idea of its molecular weight, an approximate calculation was made using the Svedberg equation and assuming a spherical particle with low hydration and a partial specific volume equal to the reciprocal of the buoyant density of the original membrane[6]. This leads to an estimate of 40 ± 4 Å for the radius and a molecular weight of 160000 ± 40000.

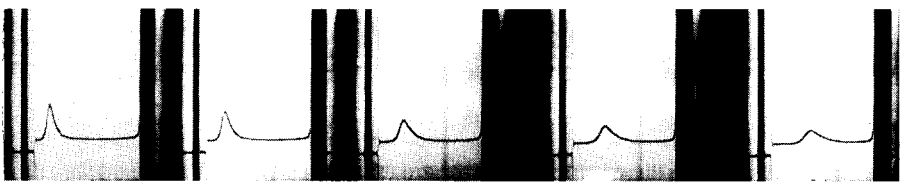

Fig. 3. Sedimentation of the lipoprotein particle in the analytical ultracentrifuge. A sample of lipoprotein particles in solution was centrifuged at 50740 rev./min and observed using Schlieren optics. Photographs were taken at 16-min intervals except that the interval between the first two photos was 8 min. A single sedimenting peak is seen with a sedimentation coefficient of 4.8 S. Small amounts of larger aggregates may be indicated by the irregularities in the line preceding the peak.

Several attempts were made to visualize the lipoprotein particle using the negative staining technique of BRENNER AND HORNE[7]. Samples of the particles (approx. 0.2 mg protein per ml) were mixed with equal volumes of 2 % solutions of phosphotungstic acid neutralized with KOH. The material was then placed in drops on Formvar-coated grids and the excess material was blotted off with filter paper after 2 min. The grids were then examined in the electron microscope. No structures appearing as particles or sheets were seen in the three attempts using negative staining. It is possible that the concentration of particles was not appropriate or that the unfixed particle is broken down in the phosphotungstic acid solution. Another possibility is that the structures might break down on drying since they are apparently stabilized by hydrophobic interactions.

Aggregation of the lipoprotein particle

Dialysis of a solution of the lipoprotein particles described above against buffer containing 0.02 M Mg^{2+} results in an increase of turbidity which is retained upon removal of excess magnesium. Centrifugation of the dialysate under the conditions usually employed for the sedimentation of membrane produced a very loose pellet, so it was decided to increase the centrifugal field used. Samples of the lipoprotein solution and the dialysate were centrifuged in the Spinco 50 Ti rotor at 35000 rev./min for 15 min. A very small pellet was observed in the tube with the lipoprotein solution and a larger pellet was seen in the tube containing dialyzed material. Both pellets

TABLE I

PARTITION OF LIPID AND PROTEIN BETWEEN PELLET AND SUPERNATANT UPON CENTRIFUGATION FOR 30 min AT 100000 × *g*

The behavior of the small lipoprotein aggregates before and after dialysis against Mg^{2+}-containing buffer is shown.

	100000 × g supernatant	*100000 × g pellet*	*% in pellet*
No Mg^{2+} control			
Protein (mg)	1.51	0.38	20
Phospholipid (counts × 10^{-6})	0.98	0.19	17
Mg^{2+} dialysate protein (mg)	0.16	1.15	88
Mg^{2+} dialysate phospholipid (counts × 10^{-6})	0.15	0.78	84

were translucent and yellow. An analysis of the distribution of protein and lipid was performed, and the results are presented in Table I.

It is clear that aggregation of the lipoprotein material into larger pieces is taking place. The sedimentation of a portion of the lipoprotein material may reflect a certain degree of spontaneous aggregation. Since the material had been standing for 2 weeks at 4° while manipulations of dialysis were being carried out to produce the aggregated lipoprotein, a slow spontaneous aggregation might have such an effect. In any event it is evident that the dialysis against Mg^{2+} accelerates the reaction. To compare the aggregated lipoprotein material with the lipoprotein particle and the original membrane from which they were derived, the protein and lipid content of three samples was determined. The samples were a suspension of the original membrane, a solution of lipoprotein particles, and a suspension of the aggregated lipoprotein material pelleted at 100000 × *g*. The data are presented in Table II.

TABLE II

PROTEIN TO LIPID RATIOS FOR VARIOUS FRACTIONS DERIVED FROM A SINGLE PREPARATION

	Protein (mg/ml)	*Phospholipid (counts/ml × 10^{-6})*	*Protein/lipid (arbitrary units)*
Original membrane	2.42	1.58	1.53
Small lipoprotein aggregate	1.81	1.18	1.54
Mg^{2+}-aggregated material	1.15	0.78	1.48

The protein to lipid ratios are remarkably constant in the different preparations, the only exception being those experiments where there is appreciable loss of lipid during dialysis without Mg^{2+}. To assess the state of the aggregated material, it was decided to observe samples in thin section using the electron microscope. Pellets of the material were formed by centrifugation at 100000 × *g* for 15 min, and were then prepared for examination with the electron microscope. The appearance of the material is seen in Fig. 4. The field is quite uniform and can be interpreted as containing small pieces of membrane with unit membrane structure visible in favorable orientations.

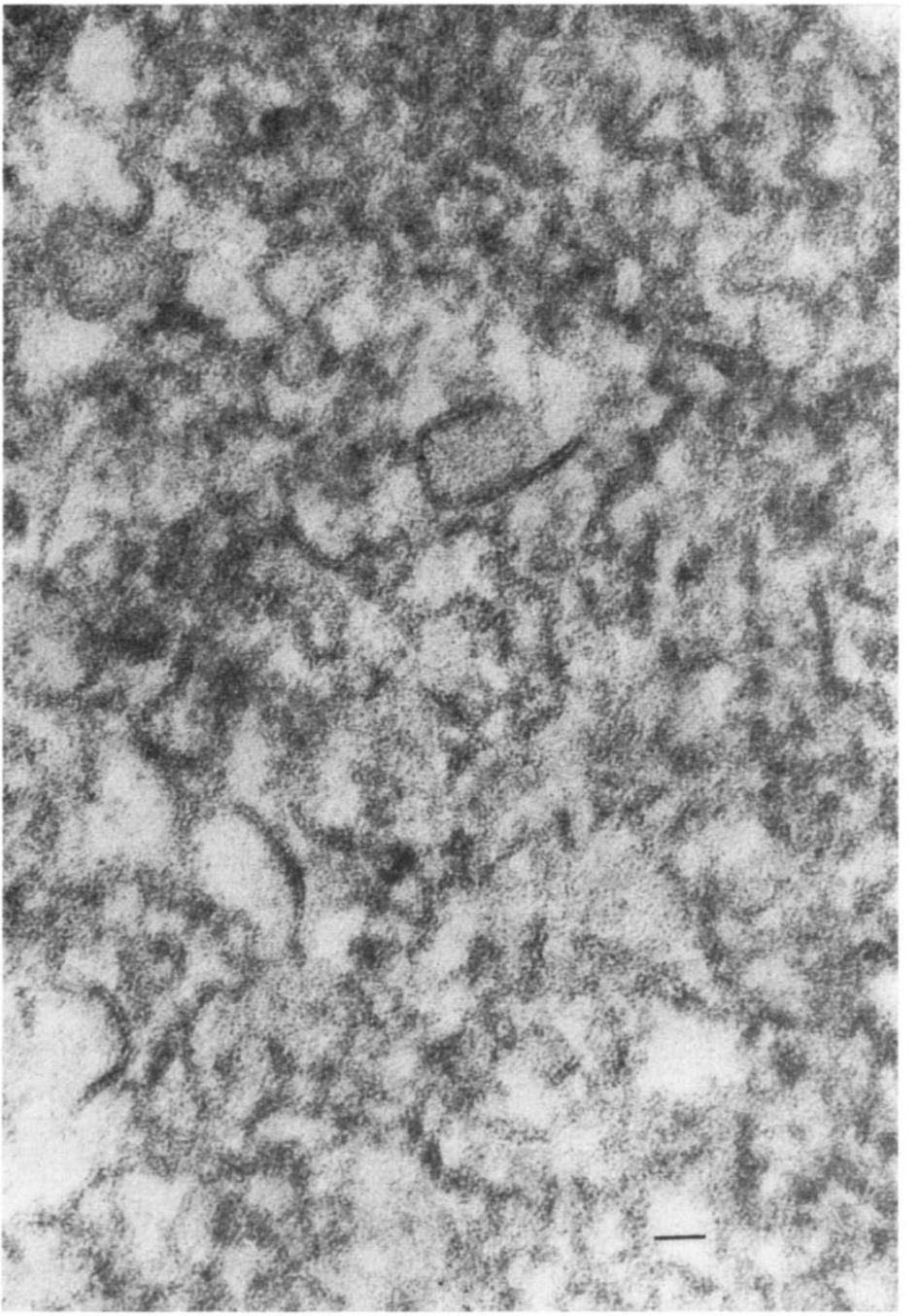

Fig. 4. Reaggregated membrane prime. A pellet of the material was fixed with formaldehyde, post-fixed with osmium tetroxide, and stained with uranyl acetate and lead citrate. Magnification is 140000 ×. The scale marker indicates 500 Å. Membranes are approx. 75 Å in thickness.

Bearing in mind the low probability of the appearance of unit membrane structure, the electron microscopic image of the aggregated lipoprotein particles may be said to be consistent with the assertion that the material is predominantly composed of small pieces of membrane. For this reason and because of the compositional data presented above, the aggregated lipoprotein material will be referred to as M_r' (reaggregated membrane prime), where "prime" is to distinguish it from the reaggregated membrane described in a previous publication[2]. More evidence supporting this terminology will be developed in the experiments which follow.

M_r' was centrifuged to equilibrium in a density gradient using the same procedures as were used in similar experiments with the original and reaggregated membranes. A sample of membrane was layered onto a 25–50 % sucrose gradient made in 1:20 β-buffer, and the gradient was centrifuged for 24 h at 35000 rev./min. Fractions from the gradient were analyzed for protein and lipid content and the resulting data are reported in the next paper in this series[8]. The reaggregated membrane prime is seen to be mostly homogeneous with a peak density of 1.18 ± 0.005 g/cm^3. Some material of lower protein to lipid ratio appears at positions just above the peak. These slightly heterogeneous elements constitute 15 % of the total material present and, since no contamination with obviously non-membranous material (such as globular aggregates) was seen in the electron micrographs, the slight heterogeneity is not considered to have great significance. The important point is that the material is mostly of fairly homogeneous buoyant density and that the mean buoyant density closely approximates that of the original membrane preparation.

The magnesium content of each of the different preparations was measured using atomic absorption and the data appear in Table III. A drop in the amount of magnesium present is seen after the original membrane is dissolved and dialyzed to produce the small lipoprotein aggregate. The original amount of magnesium appears to be restored by the dialysis producing M_r'.

TABLE III

MAGNESIUM TO PROTEIN RATIOS FOR VARIOUS FRACTIONS FROM A SINGLE PREPARATION

	Magnesium (*mg/ml*) (*a*)	*Protein* (*mg/ml*) (*b*)	(*a*)/(*b*)
Original membrane	$0.68 \cdot 10^{-3}$	0.524	$1.3 \cdot 10^{-3}$
Small lipoprotein aggregate	$0.32 \cdot 10^{-3}$	0.516	$0.6 \cdot 10^{-3}$
Reaggregated membrane prime	$0.56 \cdot 10^{-3}$	0.400	$1.4 \cdot 10^{-3}$

DISCUSSION

The principal product of dialysis of sodium dodecyl sulfate-solubilized membranes against a buffer lacking magnesium appears to be a small lipoprotein aggregate of reasonably homogeneous size, which can be aggregated to membrane-like material upon dialysis against Mg^{2+}-containing buffer. The existence of these small lipoprotein aggregates shows a possible state of the molecular components but is not necessarily related to structures *in vivo*. Nevertheless, the possibility of going from a small

aggregate to a membrane-like structure should be considered in developing models for membrane biosynthesis.

In experiments with aggregation of membrane components, the possibility of a distinction between units of assembly and subunits must be recognized. The unit of assembly may be altered upon incorporation by the membrane in such a fashion as to remove it from consideration as a subunit in the membrane structure, and the subunit need not necessarily be assembled prior to appearance in the membrane.

The nature of the involvement of Mg^{2+} with the assembly of membrane is not entirely clear. Certainly Mg^{2+} is bound during the aggregation process as indicated by the rise of Mg^{2+} concentration in the material as the small lipoprotein aggregates combine to form M_r', but whether its involvement is through divalent bridging, formation of chelate complexes with diphosphatidyl glycerol, or simple charge neutralization cannot be ascertained on the basis of present data. As already noted a certain amount of combination of small lipoprotein aggregates appears to occur spontaneously on prolonged standing at 4°, and it is possible therefore that Mg^{2+} merely accelerates the reaction rather than playing a central role.

The relation of M_r' to the original membrane and to material aggregated when the initial dialysis was done in the presence of Mg^{2+} has been the subject of further investigation and is reported in the following paper[8].

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