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Isolation of myelin bodies from the kidney cortex of gentamicin-treated rats

Lennart Nässberger [1], Anders Bergstrand [2] and Joseph W. DePierre [1]

[1] *Department of Biochemistry, University of Stockholm, Stockholm and* [2] *Department of Pathology and Research Centre, Huddinge Hospital, Huddinge (Sweden)*

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Myelin bodies have been isolated from the kidney cortex of gentamicin-treated rats (100 mg gentamicin sulfate/kg body weight i.p. twice daily for 3 days) by a simple procedure involving differential centrifugation followed by equilibrium density centrifugation on a discontinuous sucrose gradient. Electron microscopy and assay of acid phosphatase suggest that the myelin bodies were obtained in virtually quantitative yield, essentially uncontaminated by other cellular structures and relatively intact. The method developed here may also prove applicable for the isolation of myelin bodies arising in connection with other drug treatments and may provide information on a number of toxic side-effects of clinical importance.

Introduction

The aminoglycoside antibiotics are polycationic aminosugars widely used in clinical treatment of severe infections with Gram-negative bacteria. These drugs are taken up and accumulated in relatively high amounts in the proximal tubuli of the kidney cortex of both human beings [1,2] and experimental animals [3–5]. The most serious and most common side-effects of aminoglycosides are nephro- [6–8] and ototoxicity [9,10].

Of interest in this connection is the observation that treatment with aminoglycosides gives rise to characteristic morphological changes in the cells of the proximal tubuli and the haircells of the inner ear. Multilayer membrane structures resembling myelin in cross-section and called therefore myelin bodies can be detected in the kidney cortex of rats as soon as 80 minutes after a single administration of gentamicin [11]. During prolonged treatment with aminoglycosides the cells of the proximal tubuli become loaded with myelin bodies and larger structures with the morphological features of lysosomes and containing a number of myelin bodies in association are also seen [12].

There have been many proposals concerning the possible involvement of myelin bodies in the toxicological mechanism by which aminoglycosides give rise to dysfunction and/or cell necrosis in the kidney cortex. These structures are composed of concentric membrane bilayers with a mean average thickness suggesting the accumulation of polar lipids [13]. Thus, it has been proposed that myelin bodies arise as a result of inhibition of phospholipase activities and, indeed, treatment with aminoglycosides has been found to reduce the activities of phospholipase A and sphingomyelinase in the rat kidney cortex [14]. Of course, such collections of membranes might also arise from increased membrane biogenesis or alterations in the membrane traffic of the cell.

In a previous study we found that intraperitoneal administration of 100 mg gentamicin/kg body weight twice daily for 3 days to male Sprague-Dawley rats led to the formation of large numbers of myelin bodies in the kidney cortex, without causing cell necrosis [15]. Upon subcellular fractionation of this tissue the myelin bodies were recovered in the nuclear and mitochondrial-lysosomal fractions [15].

The aim of the present study has been to develop a procedure for the isolation of myelin bodies from these nuclear and mitochondrial-lysosomal fractions.

Materials and Methods

Chemicals. Gentamicin sulfate was a kind gift from Essex (Stockholm, Sweden). All other chemicals were

Correspondence: L. Nässberger, Department of Biochemistry, Arrhenius Laboratory for Natural Sciences, University of Stockholm, S-106 91 Stockholm, Sweden.

also of analytical grade and obtained from common commercial sources.

Animals and treatment. Male Sprague-Dawley rats weighing 180–200 g (ALAB, Stockholm, Sweden) were acclimated to our animal room with a 12 h light - 12 h dark cycle and constant temperature and humidity for at least 48 h before initiation of treatment. These animals had free access to commerical chow (R-J from Ewos AB, Södertälje, Sweden) containing 5% fat, 24% protein and 49% carbohydrates, as well as to tap water. In order to induce the formation of myelin bodies in the kidney cortex of the rats, they were injected intraperitoneally twice daily for 3 days with 100 mg gentamicin sulfate/kg body weight.

Isolation procedures. Rats were killed by decapitation 12 h after the final injection of gentamicin and the kidneys removed immediately. All subsequent procedures were performed at 4°C. The kidney capsule was removed and the cortex carefully dissected out with a small, sharp pair of scissors. The cortex was then minced with the scissors and thereafter homogenized at a concentration of 1 g wet weight per 10 ml 0.25 M sucrose, 10 mM Tris chloride (pH 7.4) with a Potter-Elvehjem homogenizer using 6 up-and-down strokes at 440 rpm. In a few experiments 1 mM calcium chloride was added to the homogenization medium.

Nuclear and mitochondrial-lysosomal pellets were obtained by differential centrifugation at 2000 rpm ($500 \times g_{av}$) for 5 min and, subsequently, 10000 rpm ($10000 \times g_{av}$) for 10 min [15]. We have previously demonstrated that these fractions contain all of the myelin bodies and cytosegrosomes present in the kidney cortex. Both pellets were resuspended in 5 ml 0.25 M sucrose, 10 mM Tris chloride (pH 7.4) per g original wet weight of tissue, using hand homogenization.

Six two-step discontinuous sucrose gradients were formed by hand: I, 2.5 ml 0.399 M sucrose ($\rho = 1.0507$) over 5 ml 0.632 M sucrose ($\rho = 1.0810$); II, 4 ml 0.431 M sucrose ($\rho = 1.0549$) over 6 ml 0.632 M sucrose ($\rho = 1.0810$); III, 3.5 ml 0.367 M sucrose ($\rho = 1.0465$) over 5 ml 0.771 M sucrose ($\rho = 1.0990$); IV, 3 ml 0.399 M sucrose ($\rho = 1.0507$) over 6 ml 0.771 M sucrose ($\rho = 1.0990$); V, 4 ml 0.431 M sucrose ($\rho = 1.0549$) over 6 ml 0.771 M sucrose ($\rho = 1.0990$); and VI, 3.5 ml 0.530 M sucrose ($\rho = 1.0678$) over 6 ml 0.771 M sucrose ($\rho = 1.0990$). 1 mM calcium chloride was also included in the sucrose gradients in a few experiments. 9 ml each of the resuspended nuclear and mitochondrial-lysosomal fractions were layered separately onto these gradients.

These gradients were centrifuged routinely at 30000 rpm in a Beckman 60 Ti rotor ($= 90700 \times g_{av}$) for periods of 40, 60, 80, 120 or 180 min. In a separate experiment this centrifugation was performed at 40000 rpm in the same rotor ($= 161100 \times g_{av}$) for 120 min.

The milky interphase between the lower and upper sucrose layers was pipetted off. These interphases obtained separately from the nuclear and the mitochondrial-lysosomal pellets were routinely pooled to obtain more material. After dilution with 0.25 M sucrose, 10 mM Tris chloride (pH 7.4) the myelin bodies present in these interphases were harvested by centrifugation at 20000 rpm in the 60 Ti rotor ($= 40300 \times g_{av}$) and the small, yellow-white pellet resuspended in a small volume (usually around 0.5 ml) of 0.25 M sucrose, 10 mM Tris chloride (pH 7.4).

The pellets obtained following centrifugation on the discontinuous sucrose gradients were also resuspended in 0.25 M sucrose, 10 mM Tris chloride (pH 7.4).

Acid phosphatase (with β-glycerophosphate as substrate) was assayed according to Appelman and coworkers [16].

Electron microscopy. Sampling for transmission electron microscopy was performed as described previously [15].

Results

As seen in Fig. 1, the proximal tubuli cells of the kidney cortex in rats treated with gentamicin according to our dosage and schedule contained considerable numbers of myelin bodies and aggregations thereof, without showing any morphological signs of cell damage. Our earlier subfractionation and marker enzyme study of this same tissue also indicated that these cells are essentially normal [15]. Figs. 2A and 2B show that the nuclear and mitochondrial-lysosomal fractions from the kidney cortex of gentamicin-treated rats contain the myelin bodies, as also shown previously [15].

The different conditions used for the density gradient centrifugation step are described in detail under Materials and Methods. At the very top of the centrifuge tube, a thin white band presumably containing lipoproteins was seen. The fluffy white band at the interphase between the two sucrose layers proved to contain intact myelin bodies, essentially uncontaminated by other cell structures (Fig. 3). The nuclear and mitochondrial-lysosomal fractions obtained as pellets after centrifugation on the discontinuous sucrose gradient contained virtually no myelin bodies (not shown), indicating that the recovery of these structures at the interphase was essentially quantitative. All of the different discontinuous sucrose gradients which we employed gave similar patterns, but optimal recovery, minimal contamination and optimal morphological intactness of the myelin bodies and cytosegrosomes were obtained using a upper cushion of 3 ml 0.399 M sucrose ($\rho = 1.0507$), a lower cushion of 6 ml 0.771 M sucrose ($\rho = 1.0990$) and centrifugation at 30000 rpm ($= 90700 \times g_{av}$) for 60 min. These optimal conditions resemble those used previously to isolate concentric lamellar organelles from the lung [17–19]. We have repeated this isolation of

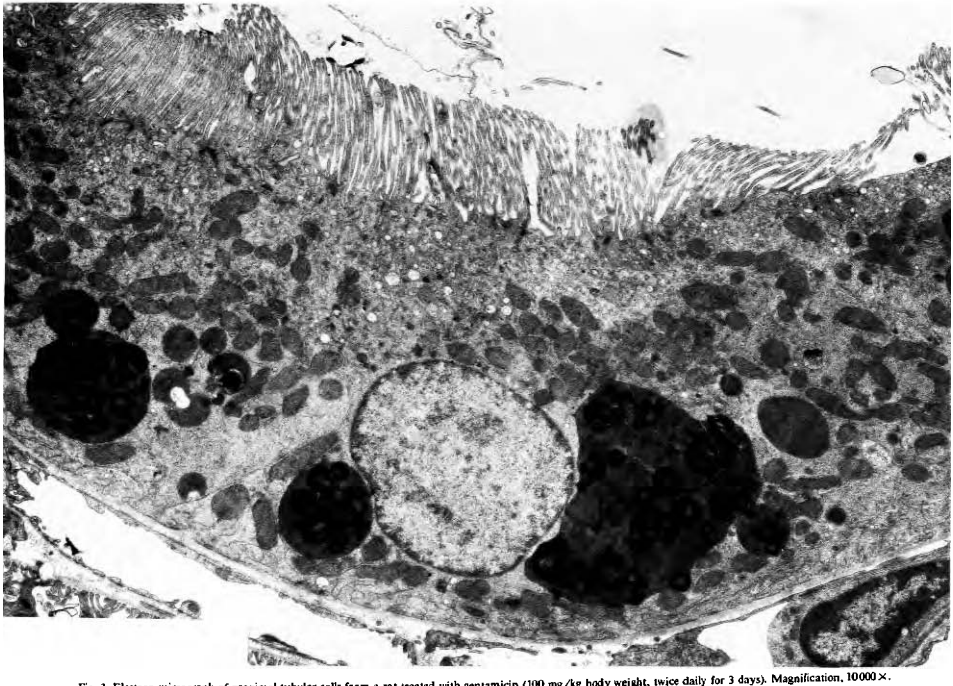
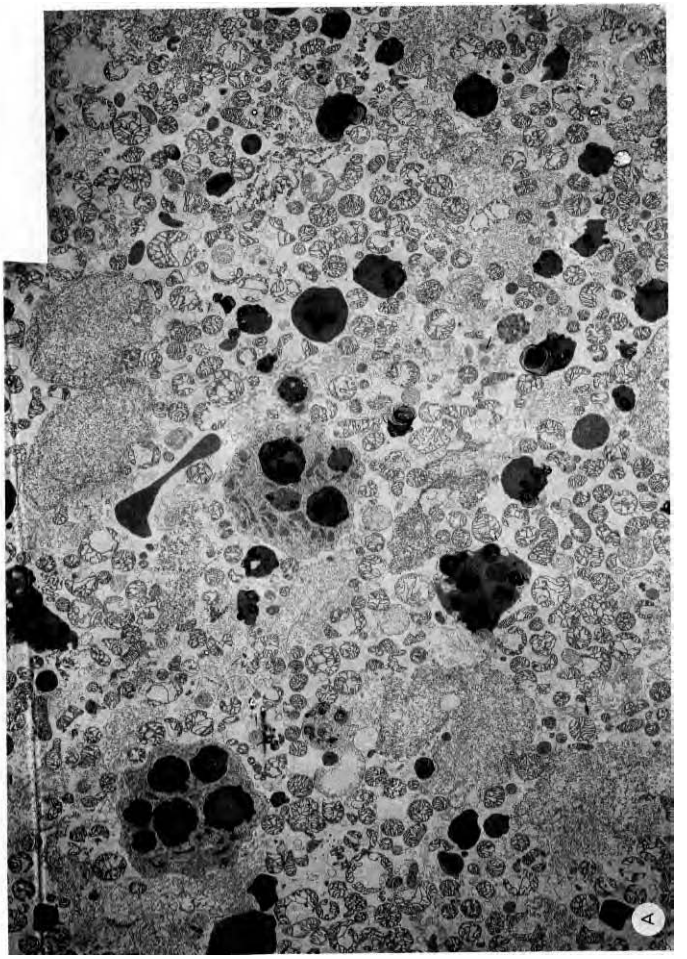


Fig. 1. Electron micrograph of proximal tubular cells from a rat treated with gentamicin (100 mg/kg body weight, twice daily for 3 days). Magnification, 10000×.



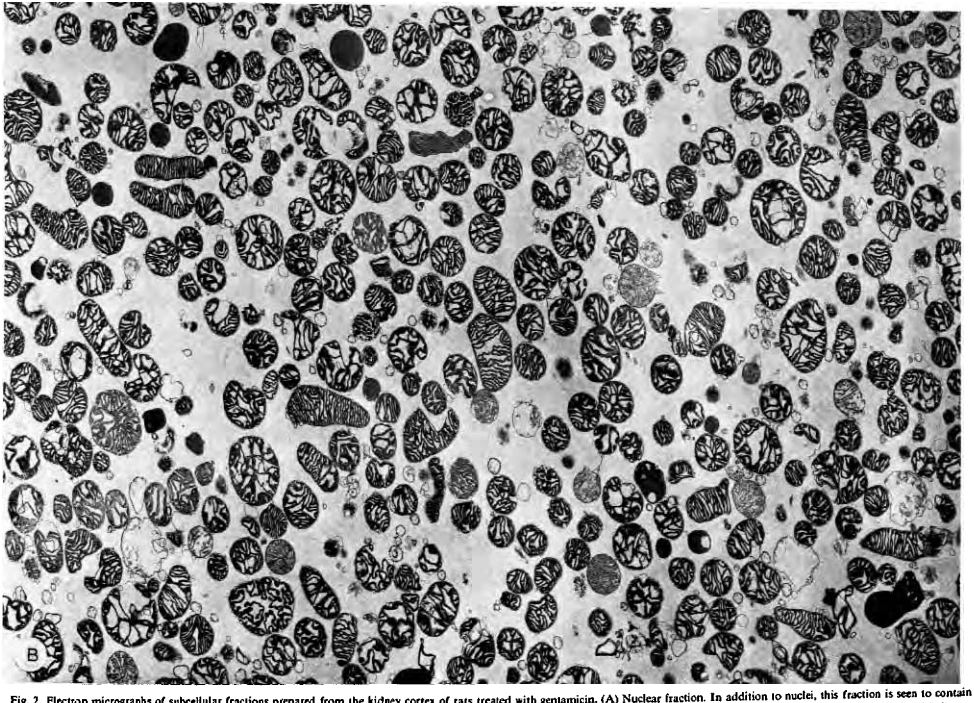
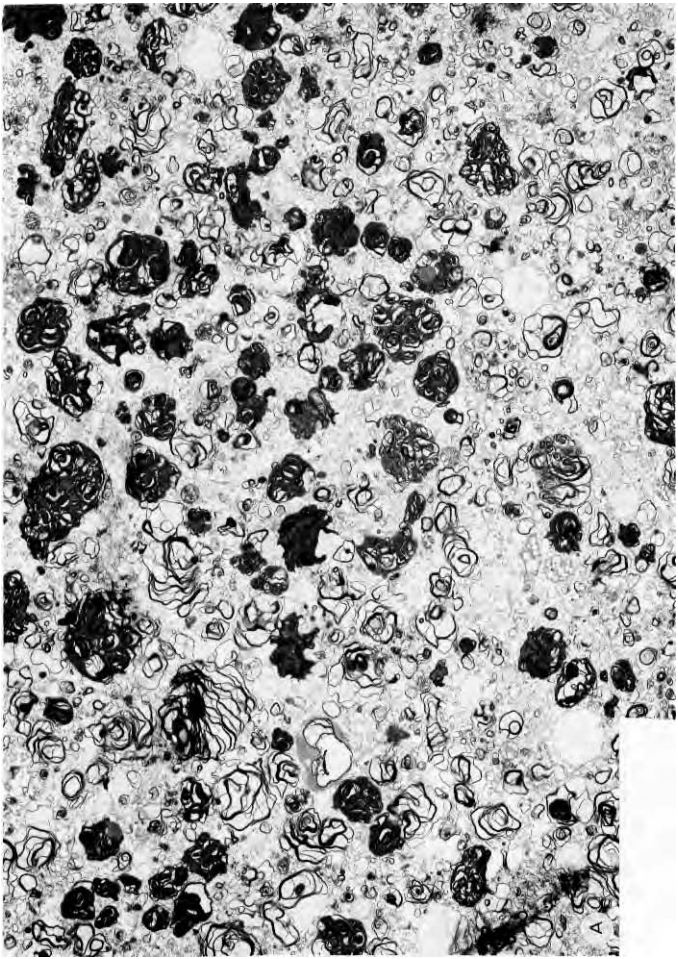


Fig. 2. Electron micrographs of subcellular fractions prepared from the kidney cortex of rats treated with gentamicin. (A) Nuclear fraction. In addition to nuclei, this fraction is seen to contain several myelin bodies and numerous mitochondria. Magnification, 5000×. (B) Mitochondrial-lysosomal fraction. This fraction contains smaller myelin bodies and some of the mitochondria have a swollen appearance. Magnification, 10000×.



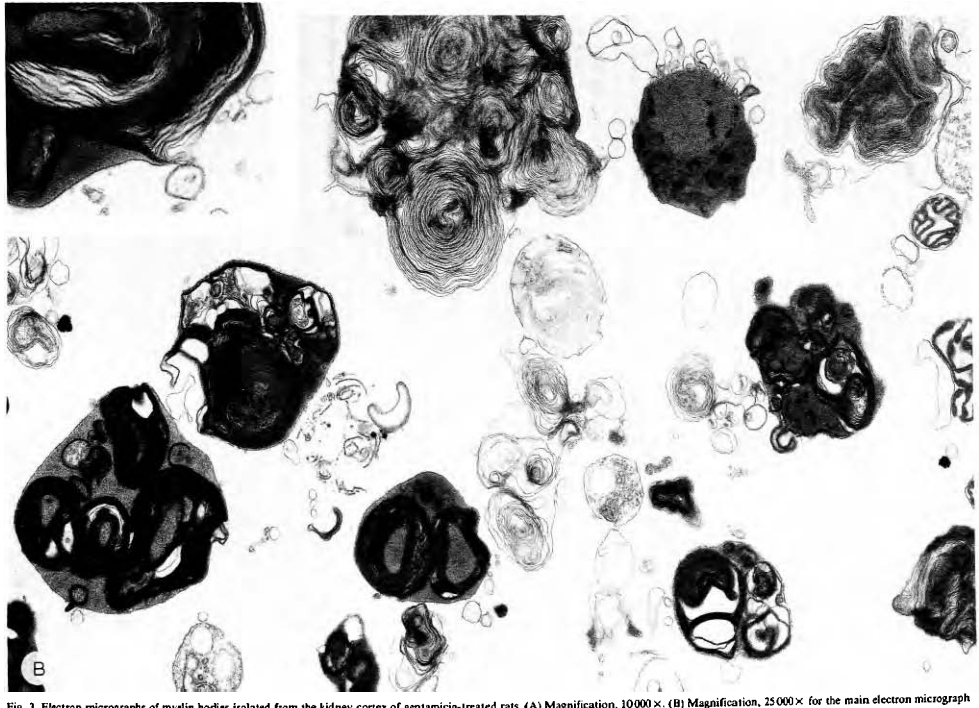


Fig. 3. Electron micrographs of myelin bodies isolated from the kidney cortex of gentamicin-treated rats. (A) Magnification, 10000×. (B) Magnification, 25000× for the main electron micrograph and 105000× for the inset.

TABLE I

Acid phosphatase activities in the upper and lower sucrose layers after density centrifugation of the nuclear and mitochondrial-lysosomal fractions, in the supernatant fraction from subsequent harvesting of the myelin bodies and in the myelin bodies themselves

Sample	Acid phosphatase (nmol inorganic phosphate/min per mg protein) in experiment number		
	1	2	3
Total homogenate	1.72	2.64	2.27
Upper sucrose layer	n.d.	n.d.	n.d.
Lower sucrose layer	n.d.	n.d.	n.d.
Supernatant fraction	n.d.	n.d.	n.d.
Myelin bodies	7.07	4.11	5.53

n.d., not detectable.

myelin bodies, with accompanying electron microscopic analysis, seven times with essentially identical results.

Some of the myelin bodies seemed to be slightly unraveled at their outer layers (Fig. 3), which led us to test other conditions which might improve the morphological intactness of these structures. However, the inclusion of 1 mM calcium chloride in the media employed throughout the isolation procedure did not affect this unraveling phenomenon or in any other way influence the morphology of the isolated structures. In addition, inclusion of 1 mM calcium chloride or 0.05% glutaraldehyde during the isolation procedure did not affect the level of radioactivity recovered in the fraction containing myelin bodies after administration of ^{3}H-gentamicin in vivo (data not shown). Thus, this antibiotic apparently does not leak out of these structures as isolated, again indicating their intactness.

In order to further test the intactness of the isolated myelin bodies we assayed acid phosphatase activity in the upper and lower sucrose layers after density centrifugation of the nuclear and mitochondrial-lysosomal fractions, in the supernatant fraction obtained from the subsequent harvesting of the myelin bodies and in these structures themselves. As seen in Table I, acid phosphatase activity was recovered in the myelin bodies at a level 2–3-times that seen in the total homogenate. In addition, no acid phosphatase activity leaked out of the myelin bodies during their isolation on the discontinuous sucrose gradient or during subsequent harvesting, again indicating their relative intactness.

Discussion

We have developed here a rapid and simple procedure for the isolation of myelin bodies from the kidney cortex of rats treated with gentamicin. After two short differential centrifugations, a single centrifugation on a discontinuous, two-step sucrose gradient yields these structures in virtually quantitative yield and essentially uncontaminated by other cellular structures. The entire isolation procedure subsequent to removal of the kidney cortexes takes about 3 h. One practical disadvantage of this procedure is that the combined cortices of the two kidneys from a 180–200 g male Sprague-Dawley rat weigh only about 1 g (wet weight) and, therefore, the yield of myelin bodies and cytosegrosomes from a single rat is small.

With this isolation method in hand, it will now be possible for us to characterize the lipid and protein-enzyme compositions of myelin bodies, as well as to quantitate in a simple manner (for example, on the basis of lipid content) these structures in the kidney cortex under different conditions. Such studies should provide valuable clues concerning the origin of myelin bodies and their possible involvement in the nephrotoxic effects of aminoglycoside antibiotics and other drugs.

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