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Sulphated Glycoconjugates in Mitochondrial Granules

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Abstract *Mitochondrial granules (MGs) are known to contain organic constituents such as phospholipids, glycolipids, glycoproteins, and lipoproteins and to be involved in the transport and accumulation of calcium ions and / or precursors of the mitochondrial inner membrane. Electron-dense mitochondrial granules have always been observed in mechanically damaged cells in cryo-fixed specimens. This study was the first to show the presence of certain sulphated glycoconjugates (SGs) in MGs, as another organic component, not only of hard tissue cells but also of soft tissue cells. Further, in ameloblasts and osteoclasts which have many mitochondria and process large amounts of calcium ions, SGs were also detected at the outer surface of the mitochondria. Although phosphate groups in MGs have been thought to contribute to calcium sequestration, this study suggested that the strongly negatively charged substances, the sulphate groups of SGs, are also involved in the accumulation of divalent cations by MGs.*

INTRODUCTION

Mitochondria in a variety of cells, chemically- or cryo-fixed, have been reported to contain mitochondrial granules (MGs). They are known as osmiophilic granules [1], elecron dense granules[2], mitochondrial dense granules[3], matrix intra-mitochondrial granules[4,5], normal matrix granules[6], or native matrix granules[7]. These granules usually contain high content of calcium and phosphorus[3,4,8] and small amounts of sulphur[9]. They have often been regarded as an artefact caused by mechanical cell damage. Phospholipids[10], glycoproteins[11], and glycidic substances[12] have been shown as organic constituents of the granules. On the other hand, based on the difference of electron density, two types of mitochondrial granules have been discussed[13] : one is an electron-dense granule containing a relatively large content of calcium; the other is electron lucent and observed after osmification, thus indicating the presence of osmiophilic phospholipids. It has also been suggested that the phospholipids give the MGs an affinity for calcium[13].

In this study, certain SGs capable of accumulating cations such as calcium and/or strontium were revealed as other organic constituents of MGs and it was suggested that the negatively charged sites play an important role for calcium precipitates within the MGs which result in the formation of electron dense MGs.

MATERIALS AND METHODS

Ten Wistar strain rats, aged 1-4 weeks, were anesthetized with 20% chloral hydrate and perfused through the left ventricle with located Ringer's solution followed by 2% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 10 min. The heart, liver, and mandibles including tooth germs were dissected and immersed in the fixative for 6 h at 4 ℃.

Detection of sulphated glycoconjugates

High iron diamine thiocarbohydrazide silver proteinate (HID–TCH-SP) technique has been shown to detect specifically sulphated glycoconjugates SGs[14].

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The fixed tissues were incubated for 18 h at room temperature in high iron diamine solution, as previously described[15]. This solution was prepared by the addition of 1.6 ml 40% $FeCl_3$ (Nakarai Chem. Co. Tokyo) to a freshly prepared diamine solution containing 120 mg of N, N-dimethyl-m-phenylene diamine (HCl) (Sigma Chem. Co. St. Louis) and 20 mg N, N-dimethyl-p-phenylene diamine (HCl) (Sigma) in 50 ml of distilled water. Control specimens were similarly incubated in a solution prepared by the addition of 1.6 ml 40% $MgCl_2$, to freshly prepared diamine solution as mentioned above, the pH of which was adjusted to 1.6 with 0.1 M HCl. These specimens were rinsed several times in distilled water, post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated and embedded in Taab 812 Resin. Some specimens were processed without post-fixation in osmium tetroxide, since it has been reported that osmification may cause non-specific precipitation of HID-TCH-SP staining under the existence of phospholipids. Ultrathin sections, cut with a diamond knife and floated onto stainless steel grids, were reacted with 2% thiocarbohydrazide (Merck, Darmstadt), in 10% acetic acid for 15 min, rinsed in distilled water, and treated with 1% aqueous silver proteinate (Merck) for 20 min in the dark. The silver proteinate background staining was eliminated by filtering (Whatman filter #2) the silver proteinate solution twice before use. Sections were then washed in 2-3 changes of distilled water. The ultrathin sections were observed without uranyl acetate and lead citrate staining.

RESULTS AND DISCUSSION

In the osmium tetroxide-fixed rat liver hepatocytes, moderate electron dense particles were found inside mitochondria (Fig. 1a, c). SGs revealed as HID-TCH-SP stain deposits were localized in accord with the mitochondrial particles (Fig. 1b, d, arrows) and inside lysosome-like structures (Fig. 1b, L). SGs were also observed associated with mitochondria of fibroblasts (Fig. 2a), cardiac cells (Fig. 2b), osteoblasts (Fig. 2c), and odontoblasts (Fig. 2d). In atrial muscle cells, SGs were detected not only in mitochondria but also in atrial specific granules (Fig. 2B, AS). It was noted that mitochondria of osteoblasts (Fig. 2c) and odontoblasts (Fig. 2d) contained less abundant SGs (Fig. 2 c, d). In ameloblasts and osteoclasts which contain many mitochondria and process large amounts of calcium, SGs were found not only inside mitochondria but also at the outer surface of mitochondria (Fig. 3a, b).

The high electron density of MGs is caused not only by mineral deposition but also by osmification. The latter is related to the presence of phospholipids in MGs and usually moderate electron dense particles appear. The presence of calcium in MGs has also been shown[4, 9, 16~18]. It has been suggested that phosphate groups of the phospholipids in MGs are involved in the sequestration of calcium by MGs[10, 19]. Although the existence of sulphur in MGs has been reported[9], nothing is known about the concrete materials including the sulphur. This study suggests that the sulphur derives from the SGs.

MGs have been observed in various types of cells processed by chemical- and/or cryo-fixation. Further, it has been suggested that the presence of calcium-rich mitochondrial granules is associated with mechanical cell damage[18]. In fact, the number of electron dense MGs increases by loading with divalent cation prior to fixation and/or by mechanical damage of cells[4]. We have also observed in high pressure rapid-frozen specimens that no MGs are seen in intact cells, but that in mechanically damaged cells, many electron dense partiles are present inside mitochondria (data not shown). Although at the physiological level, calcium concentration in cells is kept less than 10 mM, the damage to the cell membrane causes an increase in intracellular calcium concentration. Mitochondrial calcium is known to be less mobilized than non-mitochondrial cytoplasmic calcium[13], thus suggesting that the former exists in a bound state with other certain organic substances. It is most likely that the SGs in MGs play a part of calcium sequestration and that the electron dense MGs may be caused by the accumulation of large amounts of calcium ions which influx into the mechanically damaged cells. In an experience of strontium loading[20], it has been shown that strontium is accumulated inside both mitochondria and atrial specific granules of heart cardiac cells. As observed in this study, SGs are also present inside both the mitochondria and atrial specific granules of rat heart muscle cells. It is highly suggestive that the strongly negatively charged molecules, SGs in the MGs and atrial specific granules are responsible for divalent cation precipitates.

In conclusion, this study showed that MGs of

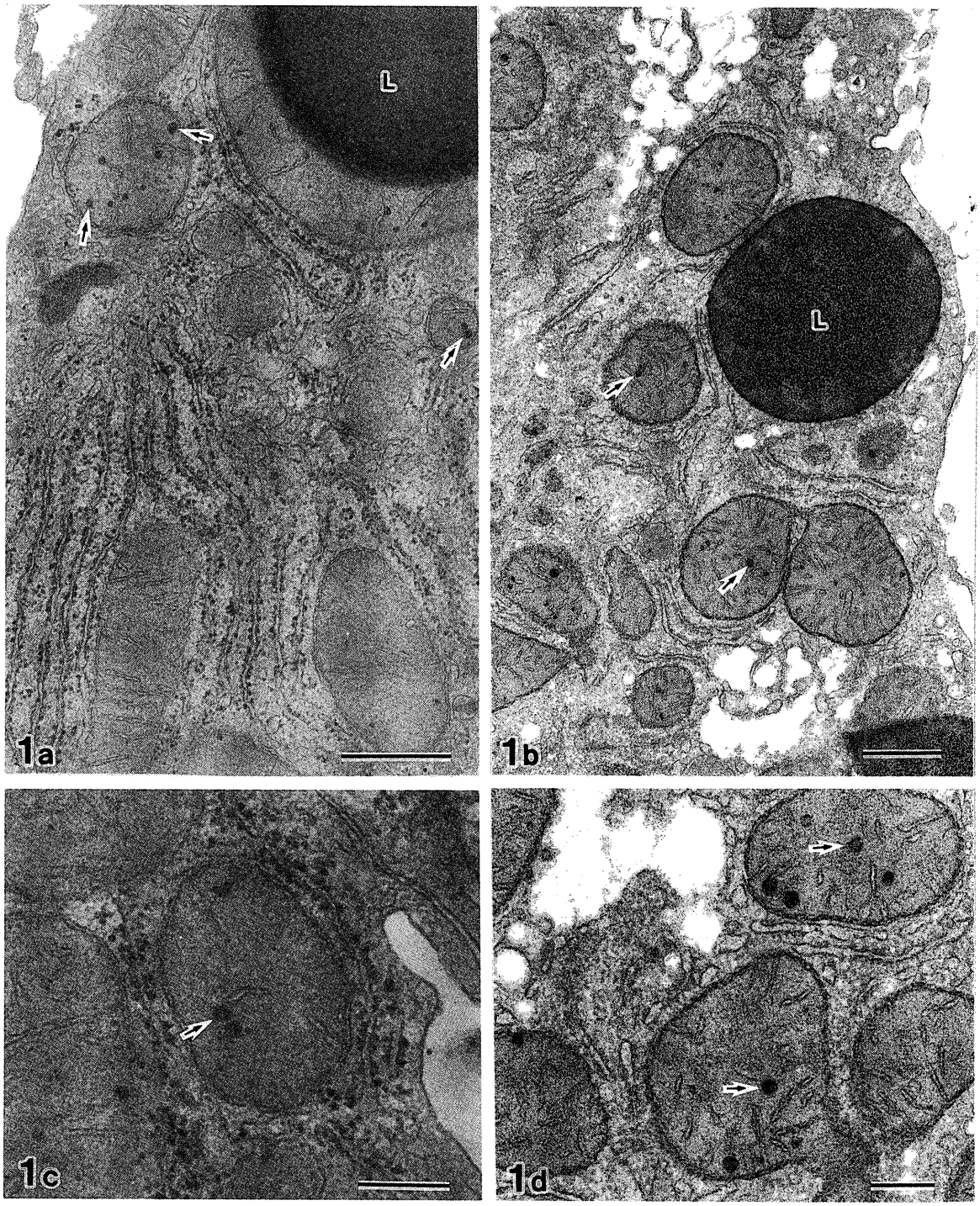

Fig. 1. **a**. Routinely prepared rat liver hepatocyte. Note moderate electron dense MGs (arrows). L : lysosome-like structure Bar= 500 nm

b. HID-TCH-SP stained rat liver hepatocyte. HID–TCH–SP stain deposits are observed in accord with the MGs (arrows). The stain deposits are also seen inside lysosome-like structures (L). Bar = 500 nm **c**. A higher magnified picture of MGs (arrow) routinely prepared. Bar = 200 nm

d. A higher magnified picture of HID–TCH–SP stained MGs (arrows). Note that the stain deposits occupy the entire MGs. Bar = 200 nm

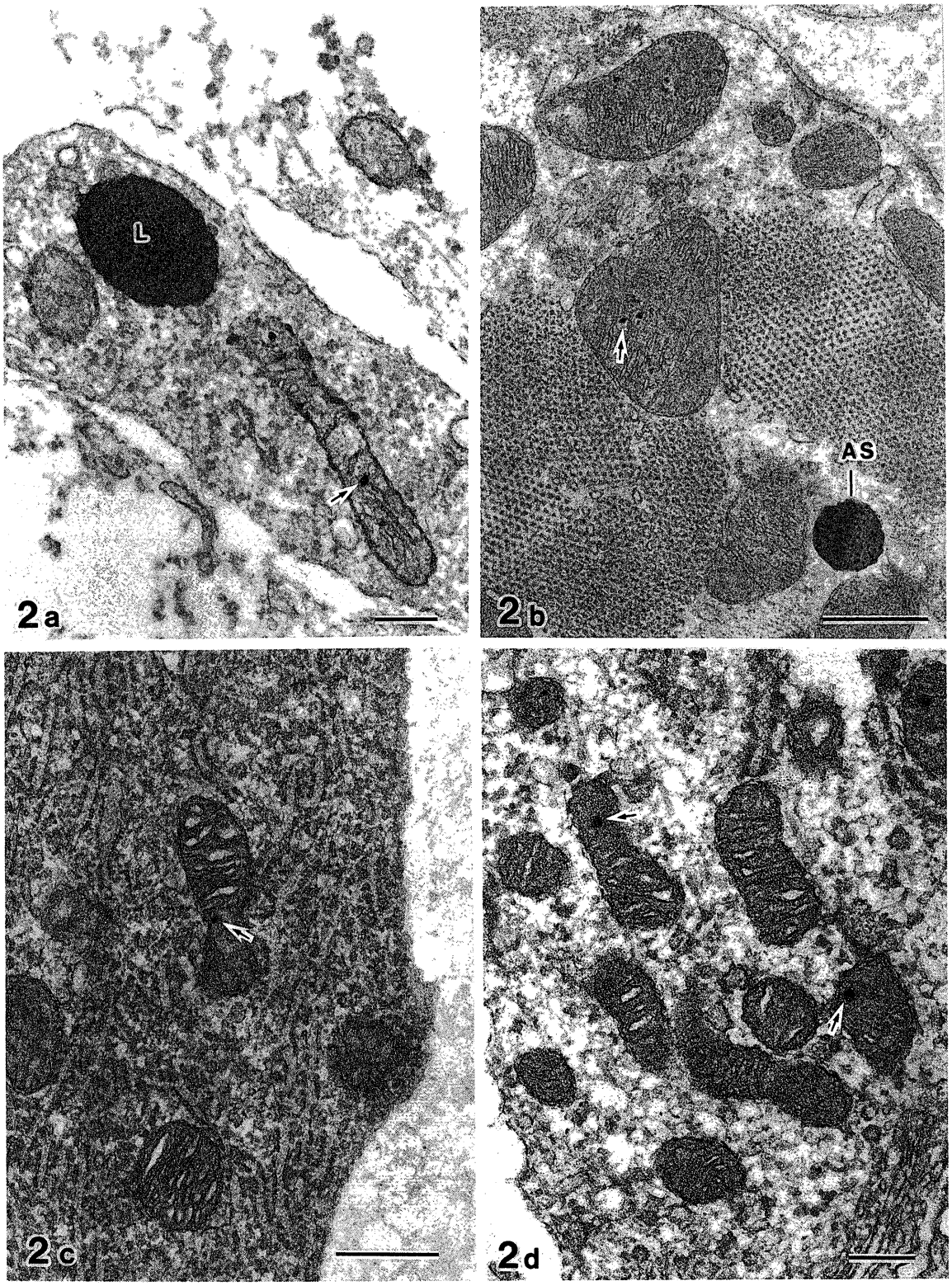

Fig. 2. Figs. 2**a**–**d** indicate HID–TCH–SP stained specimens.
a. A fibloblast in the dental follicle. The stain deposits are observed inside lysosome-like structures (L) and in MGs (arrow). Bar = 200 nm **b**. Mitochondria of heart muscle cell. The stain deposits are localized in MGs (arrow) and atrial specific granules (AS). Bar = 500 nm **c**. Mitochondria of osteoblasts in rat alveolar bone. The stain deposits are observed in MGs (arrow). Bar = 500 nm **d**. The stain deposits are localized in MGs (arrows) of odontoblasts. Bar = 200 nm

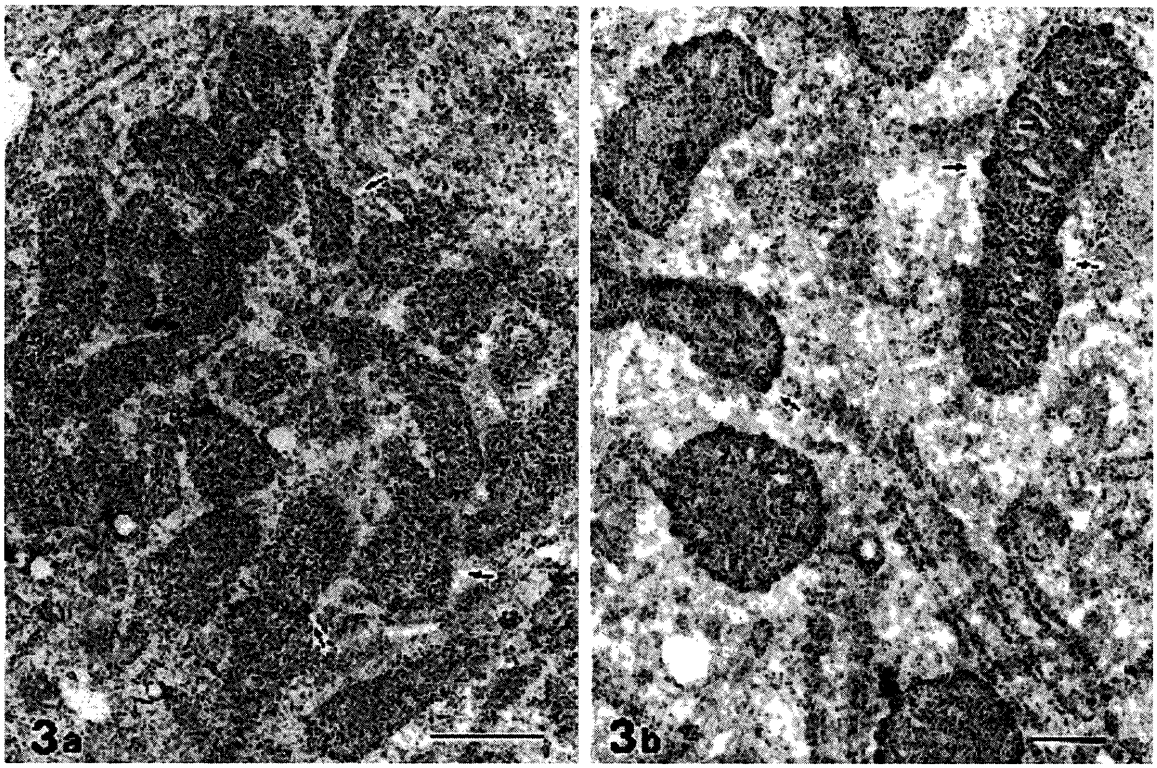

Fig. 3. Figs. 3a–b indicate mitochondria of ameloblasts **(a)** and osteoclasts **(b)** stained with HID–TCH–SP.
The stain deposits are detected not only inside mitochondria but also at the outer surface of them (arrows).
Bar = 500 nm (Fig. 3a) Bar = 200 nm (Fig. 3b)

various types of cells contain SGs, suggesting that the SGs may be involved in the accumulation of divalent cation such as calcium in mitochondria.

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ミトコンドリア顆粒の硫酸化複合糖質

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キーワード：ミトコンドリア，硫酸化複合糖質，ミトコンドリア顆粒

抄録　ミトコンドリア顆粒(MGs)は，リン脂質，糖脂質，糖タンパク，リポタンパク等の有機成分を含み，カルシウムの移送，集積ならびにミトコンドリア内膜前駆物質形成に関与することが知られている．また，凍結固定試料で機械的に損傷を受けた細胞のミトコンドリアには，常に高電子密度のMGsが現れることも報告されている．本研究は，硬組織形成細胞のみならず軟組織細胞のMGs内に新たな有機成分として硫酸化複合糖質(SGs)の存在を示した．多量のカルシウムを処理するエナメル芽細胞や破骨細胞のミトコンドリアの外表面にもSGsが検出された．従来，MGs内のリン酸基がカルシウム捕捉に関与していると報告されてきたが，本研究はMGs内の強く負に荷電したSGsの硫酸基もまた2価陽イオンの捕捉集積に関わっていることを示唆した．