J. Sci. Soc. Thailand, 9 (1983) 233 - 243

DIFFERENT PATTERNS OF INTRAMITOCHONDRIAL CALCIFICATION

SOMPHONG SAHAPHONG,[a] SUDA RIENGROJPITAK,[a] and BENJAMIN F.TRUMP[b]

a. Department of Pathabiology, Faculty of Science, Mahidol University, Bangkok, Thailand.
b. Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A.

(Received 15 August 1983)

Abstract

Using isolated rat kidney mitochondria prepared in 0.25 M sucrose medium with EDTA for maximum loading by calcium, the patterns of mitochondrial calcium uptake were studied. If the mitochondria were allowed to accumulate large amount of calcium long needle like crystals were seen, and when the lesser amount was taken up the annular pattern was observed. Therefore, it is assumed that differences in the morphology of the calcification within mitochondria from needle like crystals to annular or ringlet pattern are due to the amount of calcium taken up by the mitochondria.

Introduction

In acute cell injury especially in ischemic type, cellular calcium metabolism is disturbed. One of the most sensitive morphological indicator of effects of cell injury is the disappearence the normal mitochondrial granules and follows by accumulation of calcium within mitochondria. The accumulation of calcium is secondary to leakiness of plasma membrane and/or cessation of membrane calcium pump. In injured cells different patterns of mitochondrial calcium deposition are often observed. These include annular or ringlet images of dense particles adjacent to the inner mambrane on one hand and long-needle-like images on the other. We wish to investigate the relationship between these two configurations and the chemical composition of the calcific deposit. We propose that the pattern of calcium deposition observed may depend on the amount of calcium accumulated.

Materials and Methods

Experimental Animals Adult male Sprague-Dawley rats weighting 200-250 grams were used. They were fasted over-night and sacrificed by a rapid blow to the head, exanguinated and the kidneys quickly removed.

Isolation of Mitochondria Mitochondria were isolated in buffered 0.25 M sucrose solution by differential centrifugation according to a modified procedure of Schneider and Hogeboom[1]. The quality of preparations was checked by electron microscopy and assessment of contamination of endoplasmic reticulum of other membranes by measuring glucose-6--phosphate . The yield of mitochondrial protein was also assessed as percentage of control in relationship to renal tissue weight.

The isolation mixture consisted of 0.25 M sucrose, 1 mM EDTA, 0.5 mM Tris HCl at a pH adjusted to 7.2. Minced kidneys were homogenized in a volume of isolation mixture 5 times the renal weight. Serial centrifugations were carried out at 4°C for 10 min at 600 x g and 2 times for 15 min at 800 x g. Final resuspension in 0.6 ml sucrose per gram tissue yielded about 15 mg/ml.

Massive Calcium Loading Massive calcium loading was carried out by the procedure of Carafoli and Lehninger[2]. They differentiated two conditions for massive calcium loading. One involves a respiration supported system with succinate and betahydroxybutarate as substrate; another one uses an ATP supported system.

Incubation Mixture The incubation mixture for the respiratory dependent system contained final concentration of 4 mM sodium phosphate ($NaH_2PO_4H_2O$), 10 mM Tris-malate, 10 mM succinate, 10 mM $MgCl_2$, 3 mM ATP, 80 mM NaCl and 4 $CaCl_2$. The incubation was carried out according to the system developed by Carafoli and Lehninger[2]. The ATP-dependent system contained final concentration of 4 mM sodium phosphate, 10 mM Tris-malate, 10 mM $MgCl_2$, 80 mM NaCl, 4 mM $CaCl_2$ and 15 mM ATP. pH was adjusted to 7.0.

Incubation Procedure Incubation was carried out with 3 ml containing 4-6 mg mitochondrial protein at 30°C for 20 minutes. For time variable experiment, the concentration of $CaCl_2$ in the incubation mixture was constant at 4 mM, but the incubation intervals varied from 5 to 20 minutes, whereas, in dose variable the incubation time was fixed at 20 minutes but the concentrations of $CaCl_2$ in the medium varied from 0.5 mM to 4.0 mM.

Fixation for Electron Microscopy After completion of calcium loading the mitochondria on 0.8 Millipore filters were fixed and embedded for electron microscopy. At all times caution was taken to avoid mechanical damage by drying on filter and prolonged storage in fixative, dehydrating agent and buffer.

Under the electron microscope, at both low and high magnification, random sampling was carried out to obtain a representation sample of calcium loaded mitochondria and to estimate the amount and character of calcium loading under various conditions. Five pictures were taken from each grid at each magnification condition from the right hand corner of the first five fully covered grid fields.

Results

Control Isolated Rat Kidney Mitochondria.

Mitochondria prepared in 0.25 M sucrose exhibited a typical condensed from as described by other investigators. An example of isolated mitochondria is shown in Fig. 1. (Note the point of attachment between the inner and outer membranes).

After maximum loading for 20 minutes, all mitochondria were swollen. They contained needle-like crystals within the matrix. These needle-like crystals seemed to project from the spherical structure underneath. It is of interest to note that neither the spherical structure nor the needle-shaped crystals were seen elsewhere except within the mitochondrial matrix.

Calcium Uptake by Isolated Mitochondria : Time Variable.

In this experiment, the concentration of calcium present in the incubation medium was constant at 4.0 mM, the highest dose used in massive loading, but the incubation time was varied from 5 to 20 minutes. Electron micrographs in Figs. 2-5 show a series of mitochondria at various incubation times. After 5 minutes of incubation (Fig.2), ill-defined structures with centrally located small electron-dense dots were seen as closely related to the inner mitochondrial membrane. Electron micrographs of mitochondria incubation for 10 and 15 minutes are shown in Figs. 3 and 4 respectively. Note that the annular structures became prominent and increased in number with time. Finally, the needle-like crystals appeared at longer intervals i.e. 20 (Fig. 5).

Calcium Uptake by Mitochondria : Dose Response

Electron microscopic studies of mitochondria incubated with various concentrations of calcium chloride, 0.5 mM to 4.0 mM, revealed interesting results. With the lower concentration range of calcium 0.5 mM to 2.0 mM, incubated for 20 minutes at 30 °C, only annular patterns of densities were seen within the mitochondria. Fig.2 shows mitochondria incubated with 0.5 mM $CaCl_2$. Ill-defined structures closely related to mitochondrial inner membrane were seen. Some of these ill-defined structures contained small electron-dense dots. As the concentration of calcium chloride in the incubated medium increased, these structures developed into distinct annular bodies with more of the centrally located electron-dense spots (Figs. 8-12). The numbers of these annular bodies seemed to increase as the concentration increased. A few needle-like crystals, together with annular bodies, began to show at the concentration of 3.0 mM and became more noticeable when the concentration was further increased to 3.5 mM as shown in Fig. 12 and finally, in the so-called maximally loading experiments, more of the needle-like crystals were seen as illustrated in Fig.11.

Legends to the Figures

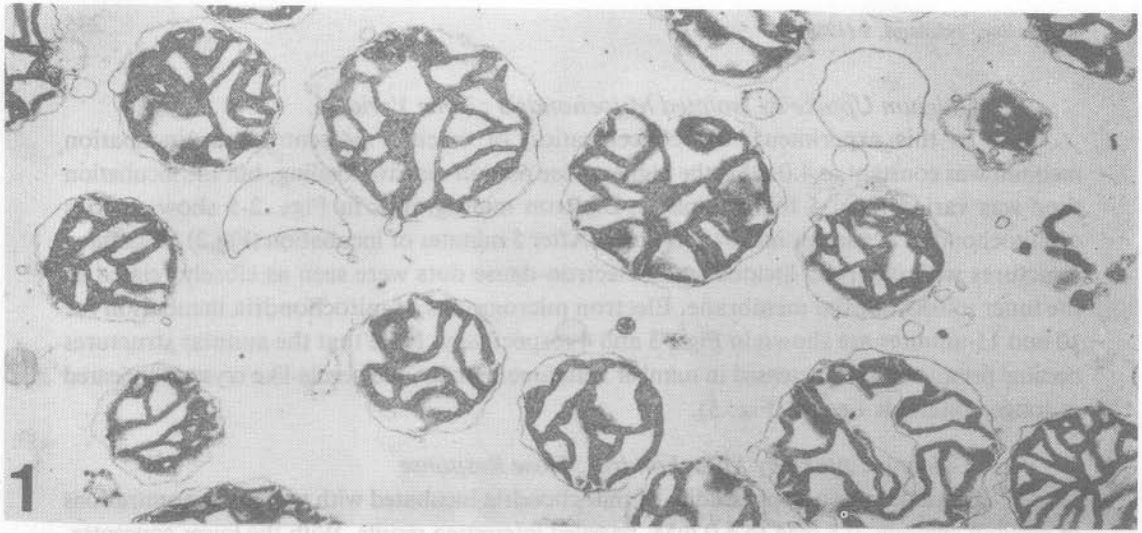
Fig.1 Control isolated rat kidney mitochondria using 0.25 M sucrose. Note the typical appearance of the mitochondrial exhibiting condensation of the matrix. x 23,000

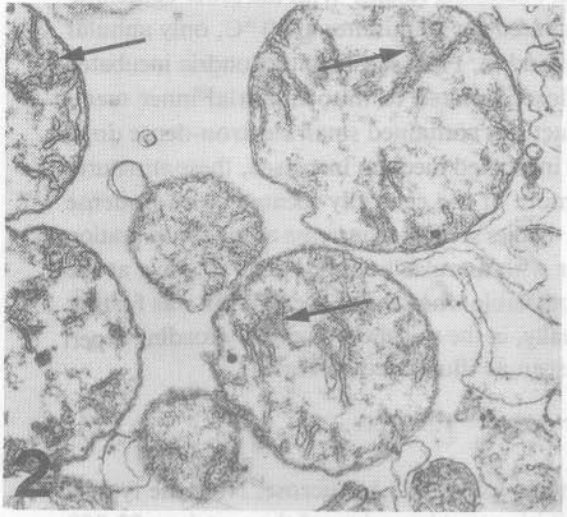
Fig.2 Mitochondria after 5 minutes incubation in massive calcium loading reaction mixture supported by externally added ATP. Note the ill-defined structure close to cristae (arrows) . x 41,000

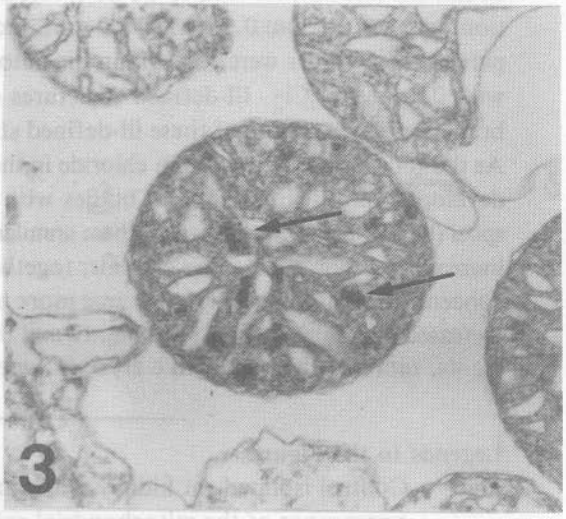
Fig.3 Same as in Fig.2. Incubation time is 10 minutes. Annular bodies (arrows) begin to appear closely ralated to the mitochondrial cristae. x 42,000

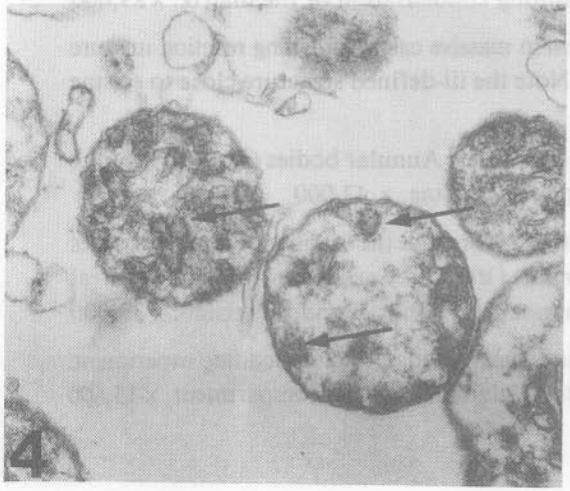
Fig.4 Fifteen minutes of calcium loading at 30 °C. Note that the number of annular bodies is increased and they are more prominent. Often they are seen with a pale central core and sometimes are located within pockets of mitochondrial cristae. x 39,000

Fig.5 Rat kidney mitochondria after 20 minutes incubation in calcium loading experiment. Long needle crystals are seen precipitated within the material compartment. x 15,600


1


2


3


4


5

Figs. 6-12 are a series of electron micrographs showing progressive formation of mitochondrial calcitification in a dose variable calcium loading experiment. In this experiment, the loading time is constant at 20 minutes. Different concentrations of calcium are present from 0.25 mM to 4.0 mM. The temperature for loading is 30°C. In this series, the uptake of calcium is supported by extremely added ATP. There is no significant difference between the two mechanisms, namely respiration or extremely added ATP,

Fig.6 $CaCl_2$, 0.5 mM. Note ill-defined structure in matrical compartment similar to 5 minutes loading in time variable experiment (Fig.2) x 43,000

Fig.7 $CaCl_2$, 1.0 mM. Annular patterns begin to appear. x 45,000

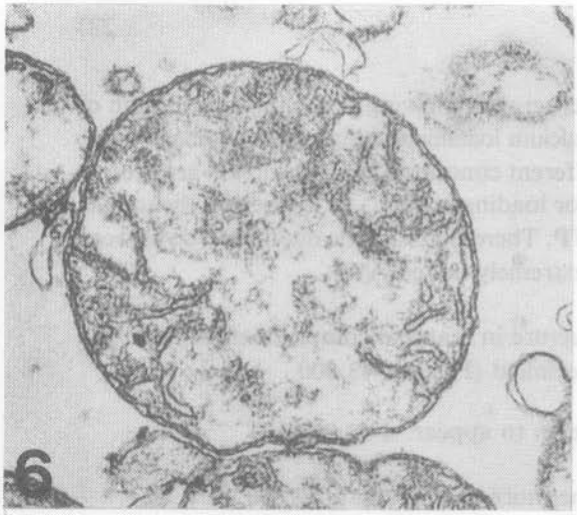
Fig.8 $CaCl_2$, 1.5 mM. These bodies become more prominent. x 59,800

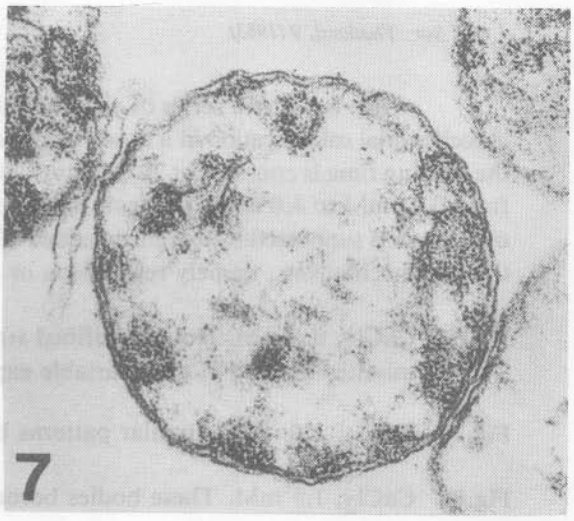
Fig.9 $CaCl_2$, 2.0 mM. Similar to Fig. 8. Note the closely embraced cristae. x 60,000

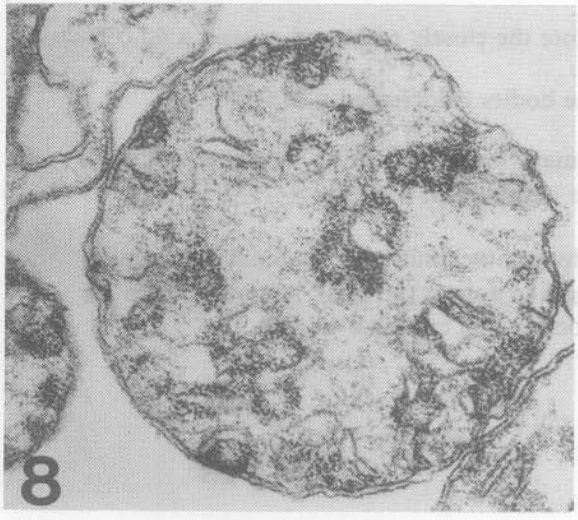
Fig.10 $CaCl_2$, 3.0 mM. The number of these bodies is increased x 38,000

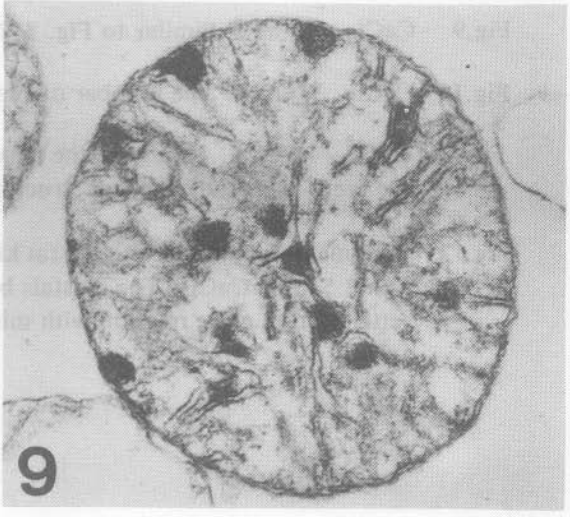
Fig.11 $CaCl_2$, 4.0 mM. Finally at the maximally loaded condition, needle-like crystals are seen. Note the spherical structure underneath. x 30,200

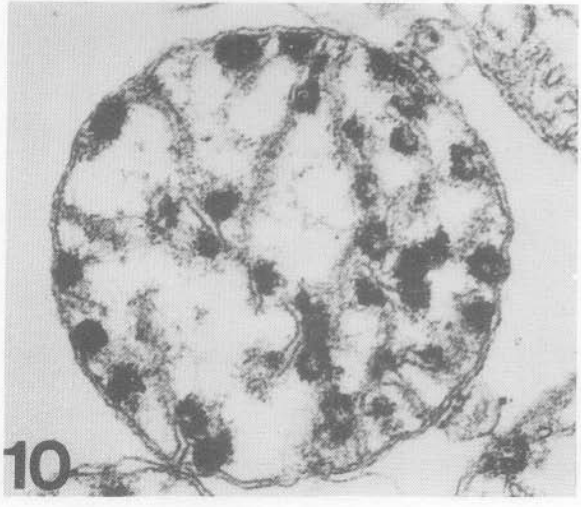
Fig.12 High magnification of isolated rat kidney mitochondria incubated for 20 minutes with 3.5 mM, $CaCl_2$. The crystals begin to appear (arrows). Annular bodies are better seen in close relation with mitochondrial criatae x 40,600

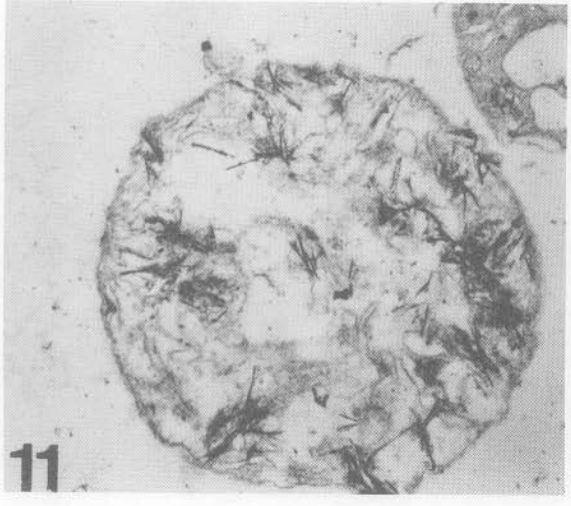

6

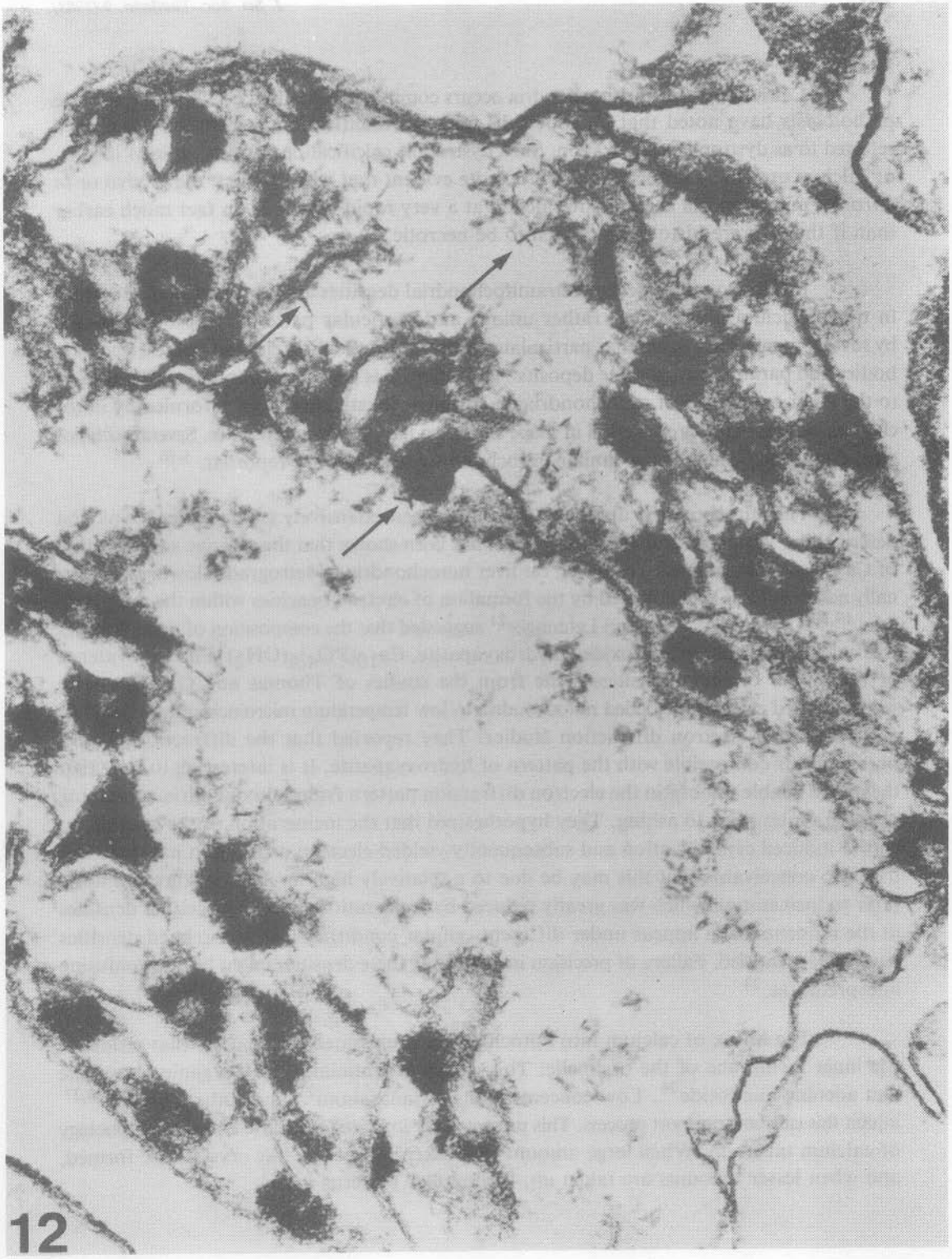

7


8


9


10


11



12

Discussion

Calcium uptake by mitochondria occurs commonly in injured cells. For a long time pathologists have noted that necrotic cells undergo calcification, which has often been referred to as dystrophic calcification. Such dystrophic calcification consists, at least in part, of calcium uptake by mitochondria. It is quite evident that when tissues die *in vivo* or *in vitro* the mitochondria accumulate calcium at a very rapid rate [3-5] in fact much earlier than if the cells are histologically seen to be necrotic.

By electron microscopy, intramitochondrial densities associated with an increase in tissue calcium uptake have rather unique and particular patterns. These are known by several names such as annular particulate bodies, ringlet bodies, circular bodies, punctate bodies and paramicrocrystalline deposits. These densities are often found near or adjacent to the inner membrane of mitochondria or, at times, located in a pocket formed by mitochondrial cristae. They are spherical in shape and often have a light central core. Several pathological conditions which show similar mitochondrial granules are reported. [5-16]

The mitochondrial uptake of Ca^{2+} has been extensively studied since it was first described by Vasington and Murphy [17,18]. It has been shown that the massive accumulation of Ca^{2+} and phosphate by respiring rat liver mitochondria or retrograde flowing of externally added ATP is accompanied by the formation of electron opacities within the mitochondria. [19,20] Greenawalt, Rossi and Lehninger[21] suggested that the composition of these densities was calcium phosphate hydroxide [hydroxyapatite, $Ca_{10}(PO_4)_6(OH_2)$]. Further evidence of the nature of these densities came from the studies of Thomas and Greenawalt[22], who subjected calcium preloaded mitochondria to low temperature microincineration (600 °C) and performed electron diffraction studies. They reported that the diffraction pattern obtained was compatible with the pattern of hydroxyapatite. It is interesting to note that they were unable to obtatin the electron diffraction pattern from mitochondria containing dense granules prior to ashing. They hypothesized that the incineration of the granules at 600°C induced crystallization and subsequently yielded electron diffraction patterns. But it is also conceivable that this may be due to a relatively high level of background noise prior to incineration which was greatly reduced by incineration. Since a variety of densities in the mitochondrial appear under different cellular conditions calcium related densities have to be identified. Failure of precision in describing these densities might lead to confusing interpretation. [23]

The influx of calcium into mitochondria is mediated by a carrier that resides in the inner membrane of the organelle. This carrier is stimulated by inorganic phosphate and adenine nucleotide[24]. Low concentrations of lanthanum[25] and ruthenium red[26,27] inhibit this calcium transport process. This present study indicated that differences in morphology of calcium taken up. When large amounts are taken up, needle-like crystals are formed, and when lesser amounts are taken up, the annular patterns occur.

Does the model of massive calcium loading correspond to actual intracellular phenomena in cell injury? Several investigators [14,28,30], using different models of cell injury, describe such situations where a defective plasma membrane does receive an excessive amount of calcium which floods the cytosol and is accumulated by still completely functioning or partially functioning mitochondria. The accumulations are commonly in the form of various types of calcium phosphate precipitates. In the earliest stage, where small amount of calcium is accumulated, aggregates form in close proximity to the crystal membranes and, at this stage, may represent amorphous aggregates which commonly have an annular profile with a clear center[5]. In later stages, however, when larger amount of calcium accumulates these precipitates become crystalline and finally replace the entire mitochondrion[31]. Such inclusions have been shown to contain calcium and phosphorus by the technique ofX-ray microanalysis and are believed to be the result of active calcium and phosphate transport by the mitochondria[32,33,34].

References.

1. Scheider, W.C.,Hogeboom, G.H. (1951) Cytochemical Studied of Mammalian Tissues: The Isolation of Cell Components by Differential Centrifugation. A Review. *Cancer Res.* **11**, 1-22.
2. Carafoli, E., Lehninger, A.L. (1978) Energy Linked Transport of Ca^{2+} Phosphates and Adenine Nucleotides. in : *Methods in Enzymology.* Oxidation and Phosphorylation. ed. by Estabrook, R.V. and Pullman, M.D., Vol. 10, pp. 745-749, Academic Press, New York.
3. Majno, G. (1964) *Death of Liver Tissue.* A Review of Cell Death, Necrosis and Autolysis, In Rouille, Ch., editor. The Liver, Morphology, Biochemistry,Physiology, Vol. 2, pp. 267, Academic Press, London and New York.
4. Trump, B.F., Croker, B.P., Jr., and Mergner, W.M. (1971) The Role of Energy Metabolism, Ion, and Water Shifts in the Pathogenesis of Cell Injury. In Richter, C.W., Scarpelli, editors, *Cell Membranes:* Biological and Pathological Aspects. p. 84, Baltimore: William and Wilkins Co.
5. Sahaphong, S., and Trump, B.F. (1971) Studies of Cellular Injury in Isolated Flounder Kidney Tubules. V. Effects of Inhibition of Plasma Membrane Sulfhydryl Groups with Organic Mercurials PCMB (parachloromercuribenzoate) and PCMBS (parachloromercuribenzenesulfonate). *Am. J.Pathol.* **63**, 277.
6. Caulfield, J.B., and Schrag, P.E. (1964) Electron Microscopic Study of Renal Calcification. *Am.J.Pathol.* **44**, 365.
7. Scarpelli, D.G.(1965) Experimental Nephrocalcinosis. A Biochemical and Morphological Study. *Lab. Invest.* **14**,123.
8. Reynolds,.E.S. (1963). Liver Parenchymal Cell Injury. I. Initial Alteration of the Cell Following Poisoning with Carbon Tetrachloride. *J.Cell Biol.* **19**, 139.
9. Zacks, S.I., and Sheff, M.F. (1964) Studies on Tetanus Toxin. I. Formation of Intramitochondrial Dense Granules in Mice Acutely Poisoned with Tetanus Toxin. *J.Neuropath. Exper. Neurol.* **23**, 306.
10. Bloom, S., and Cantilla, P.A. (1969) Myocytolysis and Mitochondrial Calcification After Low Dose of isoproterenol. *Am. J. Pathol.* **54**, 373.
11. Carafoli, E., Tiozzo, R., Pasquali-Ronchett. I., Jaschi, R. (1971) A Study of Ca^{2+} Metabolism in Kidney Mitochondria During Acute Uranium Intoxication. *Lab. Invest.* **25**, 516.
12. Hawkins, H. (1971) Lysosome Stability in Lethal Cell Injury. Ph.D. Thesis, Duke University.
13. Saladino, A.J., Bentley, P.J. and Trump, B.F. (1969) Ion Movement in Cell Injury: Effect of Amphotericin B on the Ultrastructure and Function of the Epithelial Cell of the Bladder. *Am. J.Pathol.* **54**, 421.

14. Gritzka, T.L., and Trump, B.F. (1968) Renal Tubular Lesions Caused by Mercuric Chloride. Electron Microscopic Observations: Degeneration of the Pars Recta. *Am.J.Pathol.* **52,** 1225.
15. Shen, A.D., and Jennings, R.B. (1972) Myocardial Calcium and Magnesium in Acute Ischemic Injury. *Am.J.Pathol.* **67,** 417.
16. Laiho, K.U., Shelburne, J.D., and Trump, B.F. (1971) Observations on Cell Volume, Ultrastructure, Mitochondrial Conformation and Vital-dye Uptake in Ehrlich Ascites Tumor Cells: Effect of Inhibiting Energy Production and Function of the Plasma Membrane. *Am.J.Pathol.* **65,** 203.
17. Vasington, F.D., and Murphy, J.V. (1961) Active Binding of Calcium by Mitochondria. *Fed.Proc. 20,* - 136 Abs.
18. Vasington, F.D., and Murphy, J.V. (1962) Ca^{2+} Uptake by Rat Kidney Mitochondria and Its Dependence on Respiration and Phosphorylation. *J.Biol. Chem.* **237,** 2670.
19. DeLuca, H.F., and Engstrom, G.W. (1961) Calcium Uptake by Rat Kidney Mitochondria. *Proc. Nat. Acad. Sciences, U.S.A.* **47,** 1744.
20. Lehninger, A.L., Rossi, C.S., and Greenawalt, J.W. (1963) Respiration Dependent Accumulation of Inorganic Phosphate and Ca^{2+} by Rat Liver Mitochondria. *Biochem. Biophys. Res. Commun.* **10,** 444.
21. Greenawalt, J.W., Rossi, C.S., and Lehninger, A.L. (1964): Effect of Active Accumulation of Calcium and Phosphate Ions on the Structure of Rat Liver Mitochondria. *J. Cell Biol.* **23,** 21.
22. Thomas, R.S., Greenawalt, J.W.(1968) Microincineration, Electron Microscopy, and Electron Differaction of Calcium Phosphate-loaded Mitochondria. *J. Cell. Bio.* **39,** 55.
23. Mergner, W.J., Smith, M.W., Sahaphong, S., Trump, B.F.(1977) Studies on the Pathogenesis of Ischemic Cell Injury VI. Accumulation of Calcium by Isolated Mitochondria of Ischemic Rat Kidney Cortex. *Virchows Arch B Cell Path.* **26,** 1-16.
24. Bygrave, F.L. (1978) Mitochondria and the Control of Intracellular Calcium. *Biol. Rev.* **53,** *43-79.*
25. Mela, L. (1969) Inhibition and Activation of Calcium Transport in Mitochondria. Effect of Lanthanides and Level Anestetic Drugs. *Biochemistry,* **8,** 2481-2486.
26. Moore, C.L. (1971) Specific Inhibition of Mitochondrial Ca^{2+} Transport by Ruthenium Red. *Biochem. biophys. Res. Commun.* **42,** 298-305.
27. Vasington, F.D., Gazzotti, R., Tisozzo, R., and Carafoli, E. (1972) The Effect of Ruthenium Red on Ca^{2+} Transport and Respiration in Rat Liver Mitochondria. *Biochim. Biophys. Acta.* **256,** 43-54.
28. Shen, A.C., and Jenning, R.B. (1972) Myocardial Calcium and Magnesium in Acute Ischemic Injury. *Am. J.Path.* **67,** 417-440.
29. Shen, A.C., and Jenning, R.B. (1972) Kinetics of Calcium Accumulation in Acute Myocardial Ischemic Injury. *Am. J. Path.* **67,** 441-452.
30. Thiers, R.E., Reynolds, E.S., and Vallee, B.L. (1960) The Effect of Carbon Tetrachloride Poisoning on Subcellular Metal Distribution in Rat Liver. *J. Biol. Chem.* **235,** 2131-2133.
31. Trump, B.F., Berezesky, I.K., Laiho, K.U., Osornio, A.R., Mergner, W.J., and Smith, M.W. (1980) *The Role of Calcium in Cell Injury.: Scanning Electron Microscopy/* Vol. 2, pp. 437-462, SEM, Inc., AMF O'Hare, IL 60666.
32. Ashraf, M., and Bloor, C.M. (1976) X-ray Microanalysis of Mitochondrial Deposits in Ischemic Myocardium. Virch. Arch. B *Cell Path.* **22,** 287-297.
33. Buja, L.M., Dees, J.H., Harling, D.F., and Willerson, J.T. (1975) Analytical Electron Microscopic Study of Mitochondrial Inclusions in Canine Myocardial Infracts. *J.Histochem. Cytochem* **24,** 508-516.
34. Hagler, H.G., Sherwin, L., and Buja, L.M. (1979) Effect of Different Methods of Tissue Preparation on Mitochondrial Inclusions of Ischemic and Infracted Canine myocardium. Transmisson and Analytical Electron Microscopic Study. *Lab. Invest.* **40,** 529-544.

บทคัดย่อ

ในการศึกษาเกี่ยวกับแคลเซี่ยมที่เข้าไปสะสมในไมโตคอนเดรียของไตของหนูที่แยกออกมาใน 0.2 โมลาร์ ซูโครส และมีสาร EDTA อยู่ด้วยนั้น พบว่ามีลักษณะต่าง ๆ กันตั้งแต่มีผลึกเส้นเล็กยาวคล้ายเข็ม จนถึงลักษณะกลมหรือวงแหวน ลักษณะที่แตกต่างกันดังกล่าวนี้ขึ้นอยู่กับปริมาณของแคลเซียมที่เข้าไปสะสมอยู่ในไมโตคอนเดรีย กล่าวคือถ้าไมโตคอนเดรียสะสมแคลเซี่ยมไว้เป็นจำนวนมาก แบบที่พบจะเป็นแบบผลึกคล้ายเข็ม แต่ถ้าแคลเซี่ยมที่สะสมนั้นมีจำนวนน้อยลง แบบที่พบก็จะเป็นแบบกลมหรือวงแหวน