Acta Microscópica, Vol. 1, Nº 2, November 1992, pp 27-36

Intracellular Crystallization Mechanism In Cultured Pheochromocytoma Cell Model: Insight From High Resolution Electron Microscopy

Ying-Shiung Lee and Yun-Ying Chou
Cardiovascular Division, Chang Gung Memorial Hospital,
Taipei ,Taiwan, Republic of China

ABSTRACT

Intracellular crystallization processes occurring in cultured pheochromocytoma cells induced by ascorbic acid were characterized at the atomic level by means of high-resolution electron microscopy (HREM). A series of HREM observations revealed various stages of crystallization processes occurring in the amorphous matrix of the dense bodies of the cells. By day 5 in culture the dense bodies were shown to consist of randomly packed clusters of the atom-sized dots about 0.16 x 0.23 nm in size. These dots were close-packed to form different sized clusters of very fine dot-like crystals and microcrystallites at day 7 and 10 in culture respectively. At day 14 in culture the perfect crystals in a hexagonal form were often present in the dense bodies. High-resolution images of the lattice structures in the crystals showed that the atom-sized dots about 0.18 x 0.23 nm in size were regularly arranged in a hexagonal array with center-to-center spacing of 0.26 nm. Energy dispersive X-ray microanalysis of isolated microcrystal from the cultured cells consistently detected the presence of the mineral elements such as phosphorus and calcium. X-ray mapping showed that calcium element was densely and evenly distributed in the crystals. We have succeeded in creating a cultured cell model for the investigation of intracellular crystallization processes at the atomic level. The deposition of the mineral elements, particularly calcium, has suggested to be effective for the initiation of crystallization processes or at least the transformation of amorphous nucleation phase to well-crystallized nucleation phase. It is hoped that such information obtained from the HREM observations has provided direct insight into the mechanisms of biological tissue crystallization.

KEY WORDS:
Biological crystallization, High-resolution electron microscopy, Pheochromocytoma.

INTRODUCTION

In the past decade scientists have made great efforts to investigate the processes of biological tissue crystallization [1,2]. Although exist many diverse observations,there is at present no unified theory of the mechanism of tissue crystallization. Because of the poorly-resolved X-ray diffraction analysis on the submicroscopic crystalline materials in the cells the earlier work almost concentrated on extracellular crystallization processes in the hard tissue [3-5], and ignored the mechanism of biological crystallization which may take place intracellularly. In recent years the application of high-resolution electron microscopy (HREM) to the biomedical research has provided more understanding of the biological processes by disclosing new information which was not possible to obtain with the conventional electron microscope [6,7]. We have known that with the HREM it is feasible to directly visualize the images of biological specimens at atomic resolution. Thus, the present report attempts to create a cultured cell model for the investigation of the crystallization processes occurring in the subcellular organelles at the atomic level by means of HREM. It is hoped that such information obtained from the HREM observations has provided direct insight into the mechanism of intracellular crystallization, and may be useful on the theories of biological tissue crystallization.

MATERIALS AND METHODS

Isolation and Culture of Human Pheochromocytoma Cells

Tumor tissues were obtained under aseptic conditions during surgery from patients with pathological diagnosis of adrenal pheochromocytoma. Primary cultures of pheochromocytoma cells were prepared and maintained in long-term cultures in minimal essential medium with D-valine supplemented with 15% fetal calf serum, penicillin (100 unist/ml), and streptomycin (100 µg/ml) [8-10]. Fibroblast growth was routinely inhibited by the addition of 10 µM cytosine arbinoside. The

medium was changed every second or third day. Cultures were maintained for up to 90 days and divided into the experimental groups: (1) growth medium as controls, and (2) medium containing 0.25 mM freshly dissolved ascorbic acid with glucose concentration less than 100 mg per 100 ml.

Electron Microscopy

The cells immediately after isolation and cultured cells, every two or three days, from two experimental groups were processed for electron microscopic observations. The cells were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 at room temperature for two hours, washed in several changes of 0.1 M cacodylate buffer, and postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 for two hours. The samples were dehydrated in graded concentrations of ethanol, and embedded in spurr. The ultrathin sections were stained with uranly acetate and lead citrate, and examined with a Hitachi H-500 electron microscope operated at 100 KV, and Hitachi H-9000 high-resolution electron microscope operated at 300 KV. A point-to-point resolution of 0.18 nm is attainable with the Hitachi H-9000 high-resolution electron microscope. A tilting stage was used in order to orient the specimens along the lattice directions desired with gold as a standard. High resolution images were taken under an optimal defocus condition at an original magnification of 3×10^5 to 1×10^6 times on the films.

Energy Dispersive X-Ray Microanalysis

In order to identify the presence and distribution of the mineral elements more strictly and precisely, the microcrystals in the cultured cells were isolated by mechanical disruption of the cells and centrifugation. A Hitachi S-570 scanning transmission electron microscope coupled with an energy dispersive X-ray microanalyser (EDAX-9100) was used for elementary analysis and X-ray mapping of isolated crystals mounted on the copper grids. Analytic conditions were as follows: accelerating voltage 25 KV, illuminating current 80-150 μA, specimen current 10^{-9} - 10^{-11}A, electron probe diameter 400 nm, and counting time 100 seconds.

RESULTS

In general, a series of electron microscopic observations of isolated human pheochromocytoma cells in long-term cultures with administration of ascorbic acid in the cultured medium consistently showed that the submicroscopic crystals with regular lattice structures were usually found in the subcellular organelles of the cells within two weeks in culture. The hexagonal crystal were often present in the dense bodies of the cultures cells (Fig. 1). Occasionally, the crystals were observed in the mitochondria. In contrast to the findings observed in the cells incubated in the medium containing ascorbic acid a cluster of lipid particles, instead of the crystals variable in size, appeared in the dense bodies of the cultured cells without exposure to ascorbic acid in the medium (Fig. 2). The experiments we have performed on the effect of ascorbic acid on the formation of intracellular crystals were reproducible. Various stages of crystallization processes occurring in the dense bodies of cultured pheochromocytoma cells may be identifiable a atomic resolution by means of HREM.

In order to characterize the kinetics of crystallization processes in the amorphous dense bodies of pheochromocytoma cells we sought to examine a serial changes of the microstructures of the dense bodies in the cells immediately after isolation and the in long-term cultures exposing to ascorbic acid with HREM. Electron microscopic observations of the immediately isolated cells showed that the dense bodies in the cells were always surrounded by a limit membrane (Fig. 3). These dense bodies contained patches of high electron density which were interspersed among granular moderately dense areas. High-resolution images at atomic or near-atomic level revealed that the internal structures of the dense bodies were characterized by an interweaving network of fibrillar and/or rod-shaped substructures about 0.16 nm in diameter (Fig. 4). By day 5 in cultures these dense bodies were consistently shown to consist of randomly packed clusters of the atom-sized dots about 0.16 x 0.23 nm in size. It was noteworthy that the atom-sized dots were often observed to be packed into a very short ordered segment in the filamentous network of the dense bodies (Fig. 5). Occasionally, four atom-sized dots in a group were arranged in a tetragonal form. From the 7th to 8th cultured day it was often observed that a large number of the atom-sized dots were close-packed to form different sized clusters of very fine dot-like crystals (Fig. 4). The dot-like crystals often showed regular lattice structures with interlattice spacings of about 0.12 nm. It was of particular interest that the atom-

sized dots were arranged in a hexagonal array in the crystals. Disorders in the lattice fringes were not infrequently seen. By day 10 in cultures different sized microcrystallites with regular lattice fringes were readily identified in the amorphous matrix of dense bodies (Fig. 7). At day 14 the crystals, usually in a hexagonal form, were often present in the dense bodies of the cells (Fig. 8). High-resolution images of the hexagonal crystals showed that the atom-sized dots about 0.18 x 0.23 nm in size were regularly arranged along the lattice arrays with center-to-center spacing of 0.26 nm (Fig. 9). The inter-lattice spacings were about 0.12 nm. It appeared that the atom-sized dots along the lattice arrays were arranged on the filamentous network in the amorphous matrix of the dense bodies.

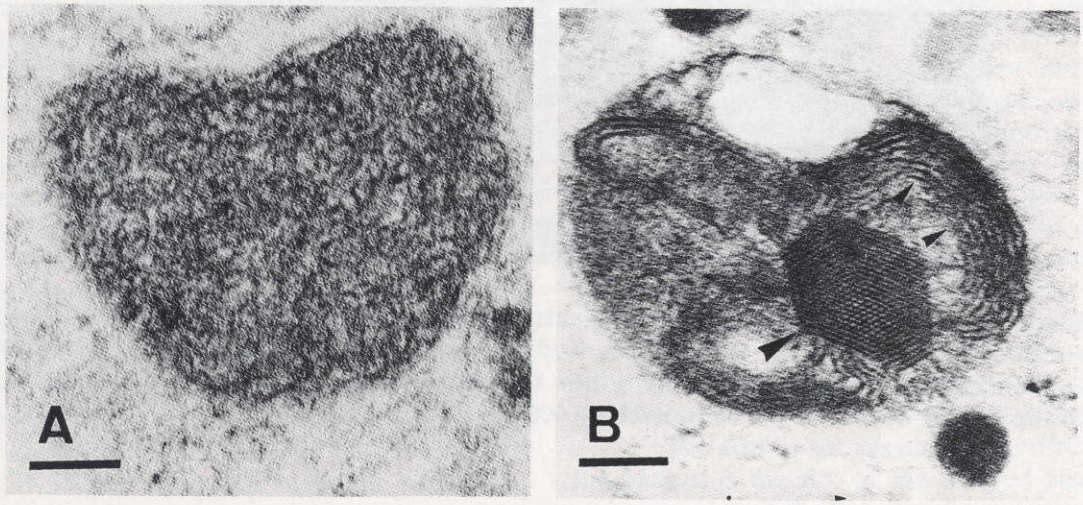

Fig. 1 Electron micrographs of the 14-day-old cultured pheochromocytoma cells. A, The dense body in the cell without exposure to ascorbic acid in the medium as the control contains an amorphous matrix with high electron density. B, The submicroscopic crystal (large arrow head) formed in the dense body of the cell with continuous exposure to ascorbic acid is observed. Note that lamellar structures are also seen in the matrix of the dense body (small arrow heads). A and B, Horizontal bar= 100 nm .

Energy dispersive X-ray microanalysis of the isolated crystals revealed many peaks of the elements such as calcium, phosphorus, sulfur, iron, silicon, sodium and potassium (Fig. 10). Although marked variation in the proportion and distribution of the individual elements from analysed site to analysed site was present, the calcium element was consistently detected from different analysed sites. Furthermore, X-ray mapping disclosed homogeneous distribution of the calcium element in the crystals (Fig .11).

DISCUSSION

Although biological tissue crystallization have been described previously [1,2], the earlier work almost concentrated on extracellular crystallization processes in the hard tissue [3-5]. It is well known that biological tissue crystallization may take place intracellularly. In the present report we have succeeded in creating a cultured cell model for the investigation of intracellular crystallization processes at atomic resolution by means of high-resolution electron microscopy. It is important to note that while various pathologic conditions may result in biological tissue crystallization, their pathogenetic origin can be quite different. Hence, the mechanism responsible for intracellular crystallization may be different in various conditions. We will therefore consider our working model of potential pathogenetic mechanism for crystallization processes occurring in the subcellular organelles of human pheochromocytoma cells in culture.

It has long been known that ascorbate is toxic to many types of the cells in culture [11]. Although ascorbate is widely used in experiments employing chromaffin cell cultures [12], there have been relatively few references for the toxicity of this compound towards such cells.

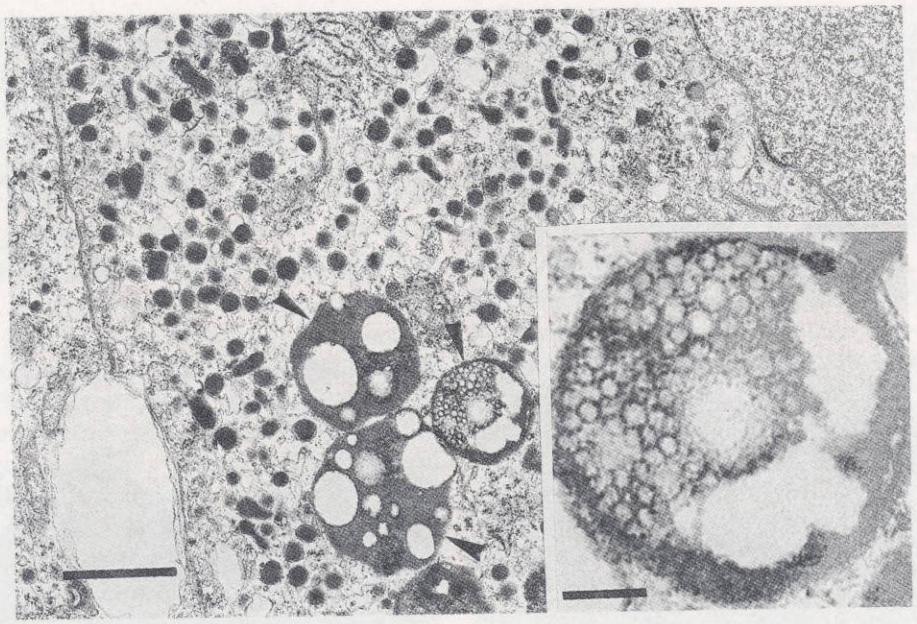

Fig. 2 Electron micrographs of the 90-day-old cultured pheochromocytoma cell without continuous exposure to ascorbic acid. Most of the dense bodies (arrow heads) contain a lipid component and irregular patches of high electron density within their matrix. Horizontal bar = 1 μm . Inset: higher magnification of the dense body. Horizontal bar= 200 nm .

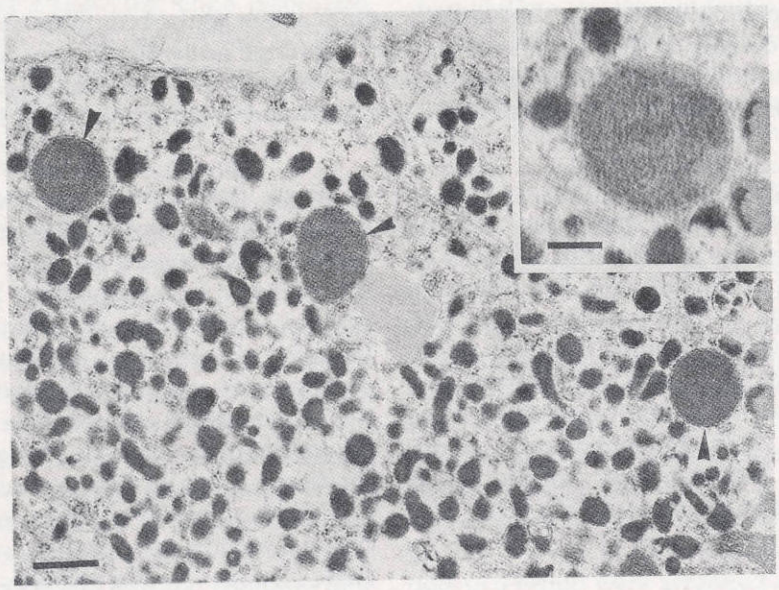

Fig. 3 Electron micrographs of the substructures of the dense bodies in human pheochromocytoma cells immediately after isolation. The dense bodies with granular matrix are always surrounded by a limiting membrane (arrow heads). Horizontal bar= 0.5 μm . Inset: higher magnification of the dense body. Horizontal bar = 200 nm .

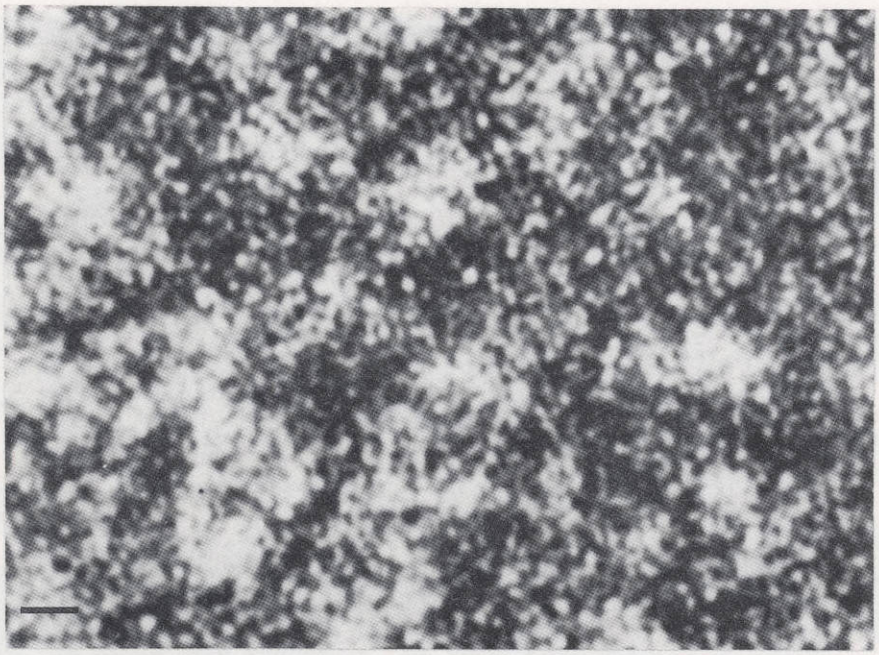

Fig. 4 High-resolution image of the dense body in human pheochromocytoma cell immediately after isolation. The rod-shaped and fibrillar substructures in the amorphous matrix of the dense body are closely packed and interconnected to form an interweawing network. Horizontal bar= 1 nm .

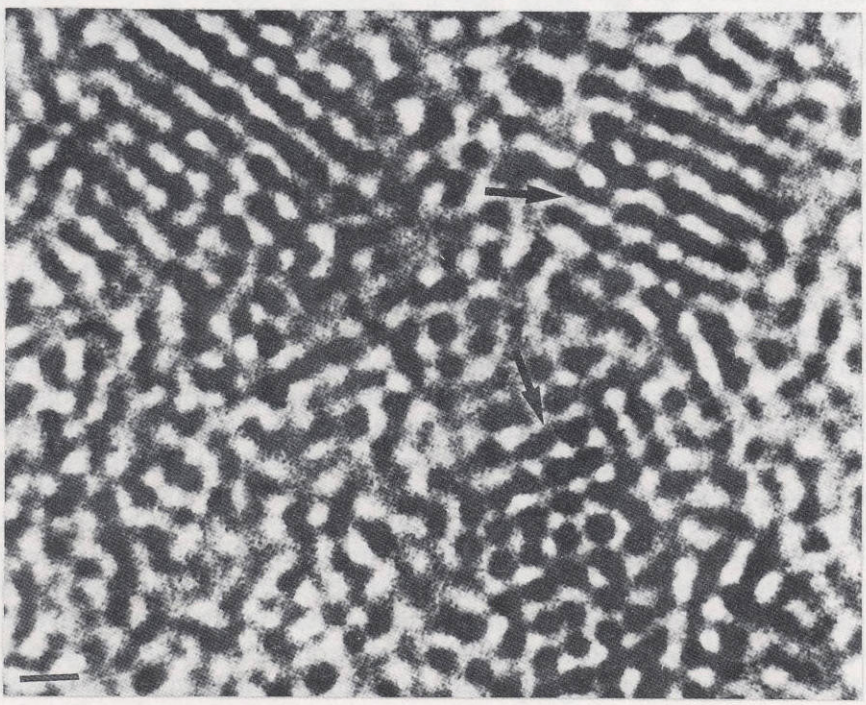

Fig. 5 High-resolution image of the dense body in the 5-day-old cultured pheochromocytoma cell incubated with ascorbic acid in the medium. The atom-sized dots are packed into very short ordered lattice-like segments (arrows) in the amorphous matrix of the dense body. Note that four dots in a group are in a tetragonal-like arrangement. Horizontal bar= 0.5 nm .

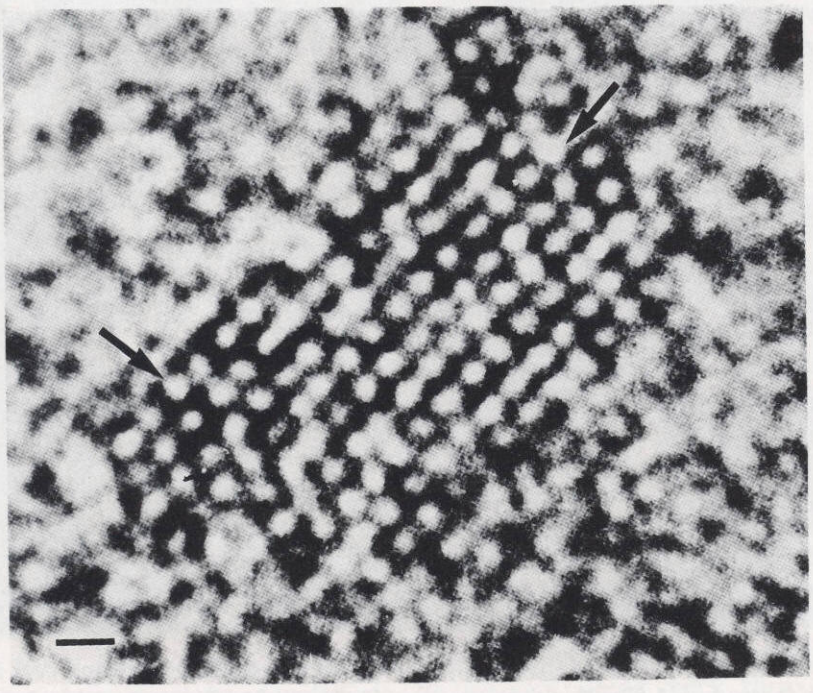

Fig. 6 High-resolution image of the dense body in the 7-day-old cultured pheochromocytoma cell with continuous treatment of ascorbic acid for 7 days. A cluster of very fine dot-like crystal with regular lattice array (arrows) is clearly identified in the amorphous structure of the dense body. Note that the atom-sized dots are arranged in a hexagonal lattice. Horizontal bar= 0.5 nm .

Repetition of the experiments we have performed consistently showed the effect of ascorbic acid on the facilitation of crystal formation in the dense bodies of cultured pheochromocytoma cells. However, the exact role of ascorbic acid in promoting the amorphous to crystalline transformation remains unclear. It is well established that cellular oxidation reactions involving ascorbate often lead to the production of oxygen free radicals such as H_2O_2. Furthermore, H_2O_2 treatment of liposomes prepared from human erythrocytes was evidenced to result in loss of polyunsaturated lipids by peroxidation concomitantly with an alterations in the permeability of the treated liposomes [13,14]. The site of action of H_2O_2 appears to be lipid component in the cell membrane. Thus, we suggest that alterations in membrane integrity and permeability resulting from peroxidation of fatty acids in the cell membrane by H_2O_2 is an important pathogenetic mechanism responsible for intracellular crystallization.

In the present study a serial observations of human pheochromocytoma cells in the long-term culture have demonstrated the crystallization processes occurring in the dense bodies of the cells incubated in the medium containing ascorbate. The morphological transformation of the amorphous to crystalline phase in the dense bodies was characterized at atomic resolution by means of HREM. There was a continuous formation of different sized clusters of atom-sized dots in or on the filamentous network in the amorphous matrix of the dense bodies. The tight connection of filamentous network with atom-sized dots suggests that the filamentous substructures in the dense bodies may play a structural and/or regulatory role in the mediation of the amorphous to crystalline transformation. Initially, these atom-sized dots randomly distributed in the amorphous matrix of dense bodies of the cells were arranged in a tetragonal-like form by day 5 in culture. Subsequently, very fine dot-like crystals with a hexagonal lattice appeared in the amorphous dense bodies of 7-day-old cultured cells. The atom-sized dots in a tetragonal arrangement may represent the amorphous precursors while the dot-like crystals with hexagonal lattice indicate the earliest crystals, called critical crystal nuclei. It was noteworthy that the arrangement of atom-sized

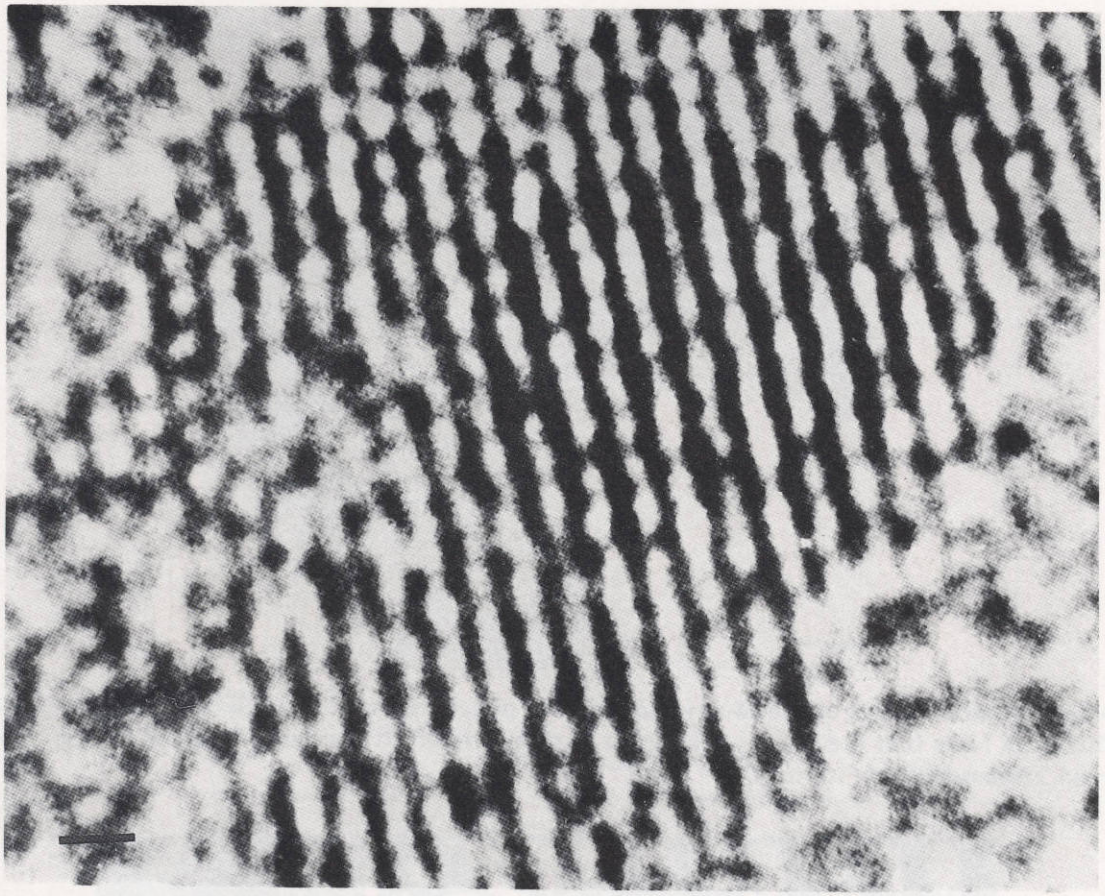

Fig. 7 High-resolution image of the dense body in the 10-day-old cultured pheochromocytoma cell with continuous exposure to ascorbic acid in the medium. The microcrystallite with regular lattice fringes is clearly observed in the amorphous matrix of the dense body. Horizontal bar= 0.5 nm .

dots in the amorphous precursors was quite different from that in the crystal nuclei. This finding indicates that the rearrangement of the constituent atoms in the dense bodies occurs in the transformation of amorphous precursors to crystal nuclei. Since the (atomic) lattice disorders were often present in the crystal nuclei the most distinguishing feature of the crystal nuclei is its lack of crystal and chemical perfection in addition to small crystal size. With progressive time in culture these earliest crystals act as nucleators for more crystals increasing in average size to maturity and the total number of crystals in the dense bodies of the cells. After two weeks in culture we did not observe lattice disorders in the hexagonal crystals in the dense bodies of the cells. Thus, concomitant with the maturation of crystal size is an improvement in chemical perfection.

It is well known that calcium element plays an important role in biological mineralization and crystallization in a variety of pathological conditions. In the present study X-ray microanalysis of isolated microcrystals from the cultured cells consistently showed that the presence of calcium in the crystals is the highest proportion in comparison with other elements. Furthermore, X-ray mapping revealed that calcium element was densely and evenly destributed in the crystals. These findings indicate the close association of calcium element with crystal formation in the dense body of the cultured cells. As discussed above, the alterations of the cell membrane permeability resulting from the detrimental effect of oxygen free radicals produced by cellular oxidation reaction involving ascorbic acid can augment the influx of extracellular calcium into the cells. The increased influx of calcium would raise intracellular calcium concentration to reach levels at which calcium would precipitate spontaneously in the matrix of the dense body of

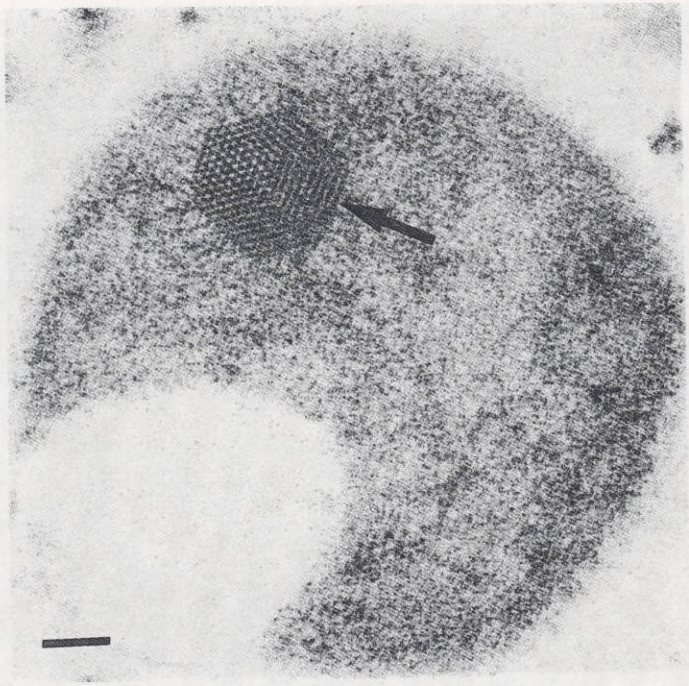

Fig. 8 Electron micrograph of the 14-day-old cultured pheochromocytoma cell continuously exposed to ascorbic acid. Note that the hexagonal crystal (arrow) formed in the dense body of the cell is clearly observed. Horizontal bar= 100 nm .

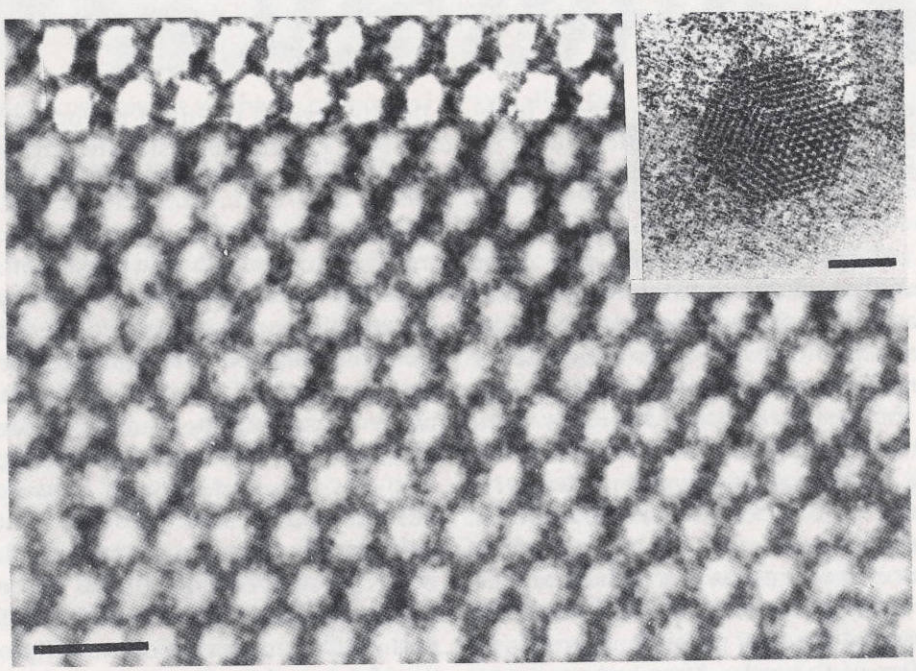

Fig. 9 Ultrahigh resolution image of the microcrystal (inset) in the dense body of the 14-day-old cultured pheochromocytoma cell incubated with ascorbic acid in the medium. The hexagonal arrangement of the atom-sized dots along the lattice arrays is clearly demonstrated. Horizontal bar= 0.5 nm . Horizontal bar (inset)= 100 nm .

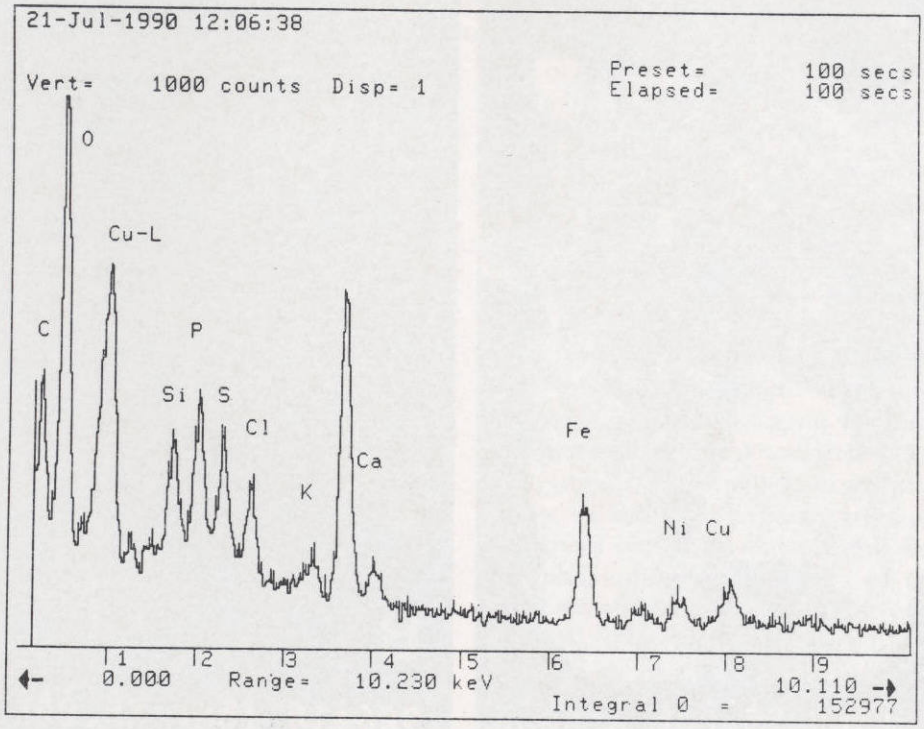

Fig. 10 Energy-dispersive X-ray microanalysis of the crystals isolated from cultured human pheochromocytoma cells showing the presence of calcium in a larger proportion compared with other elements such as phosphorus, sulfur and iron.

the cells. The proteolipid complexes which are present in the dense body as a structural skeleton serve as organic substrates by providing sites for calcium deposition with subsequent formation of critical crystal nuclei. Once the crystal nuclei are formed and undergo growth by joining with other nuclei the final crystals may or may not remain attached to the substrate macromolecules.

In summary, we have succeeded in creating a cultured cell model for the investigation of intracellular crystallization processes at the atomic level. The experiments which we have performed of the effect of ascorbic acid on the formation of the crystals in the subcellular organelles of cultures pheochromocytoma cells lend support to the supposition that the deposition of calcium in the cells due to calcium overload is a prerequisite for the initiation of crystallization processes or at least the transformation of amorphous nucleation phase to well-crystallized nucleation phase with subsequent formation of intracellular microcrystals.

REFERENCES

1. Posner, A.S., Betts, F. and Blumenthal, N.C. (1980) Formation and structure of synthetic and bone hydroxyapatites. Prog. Crystal Growth Charact. **3:** 49-64.

2. Posner, A.S. and Betts, F. (1981) The chemistry and biology of mineralized connective tissues. (ed A. Veis) Elsevier North Holland Inc. pp. 257-266.

3. Blumenthal, N.C. and Posner, A.S. (1973) Hydroxyapatite: Mechanism of formation and properties. Calc. Tiss. Res. **13:** 235-243.

4. Irving, J.T. (1973) Theories of mineralization of bone. Clin. Orthopaed. 97: 225-236.

5. Howell, D.S. and Pita, J.C. (1976) Calcification of growth plate cartilage with special reference to studiess on micropuncture fluid. Clin.

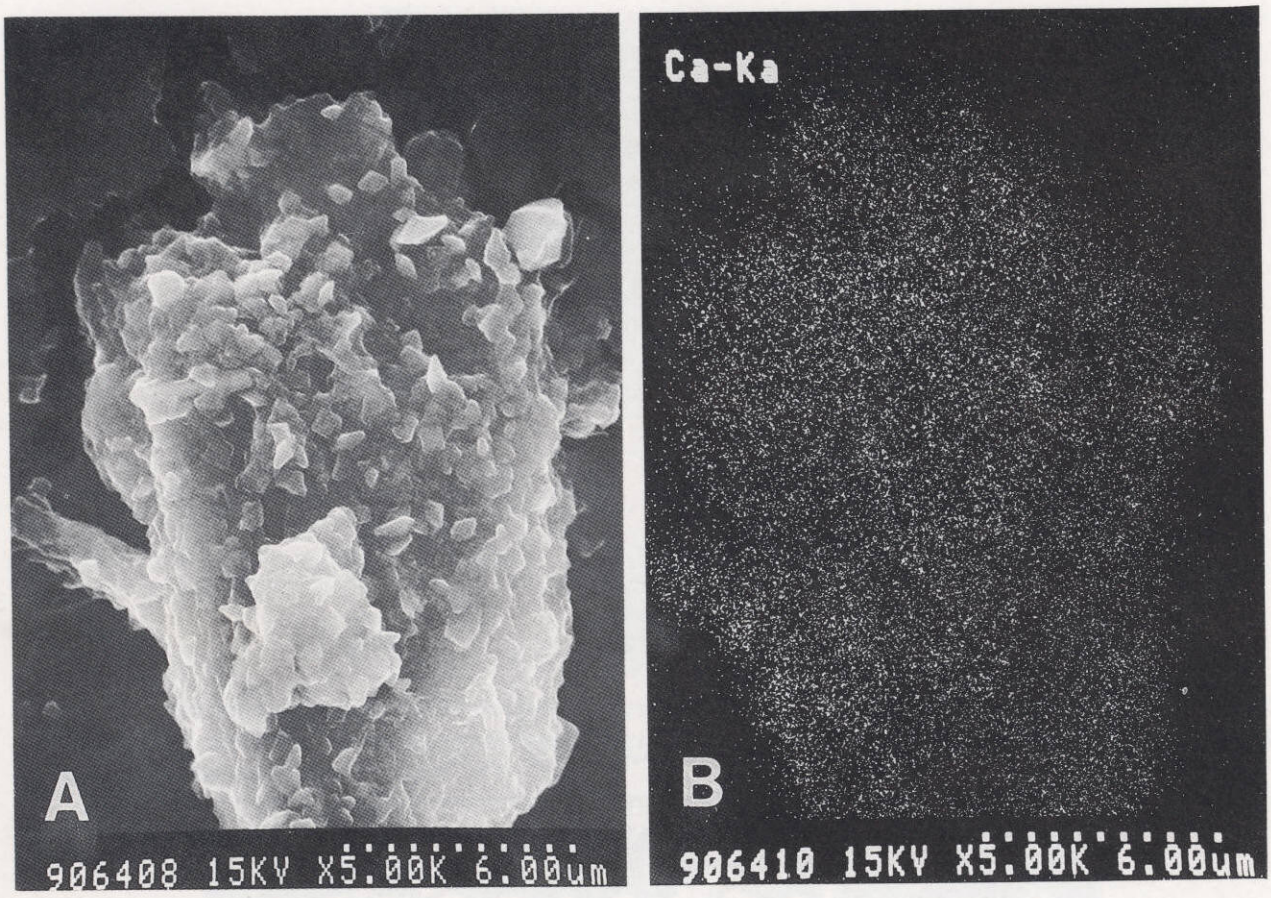

Fig. 11 X-ray mapping of calcium element in the crystals (A) isolated from cultured human pheochromocytoma cells showing a dense and even distribution of calcium in the crystals.

Orthopaed. **118**: 208-229.

6. Lee, Y.S. (1987) High resolution electron microscope lattice images of powdered gallstones. Micron Microsc. Acta **18**: 281-290.

7. Lee Y.S. (1990) High-resolution electron microscopic investigation of crystalline cholesterol monohydrate in human atheroma at the atomic level. Micron Microsc. Acta **21**: 1-12.

8. Livett, B.G. (1984) Adrenal medullary chromaffin cells in vitro. Physiol. Rev. **64**: 1103-1160.

9. Jaques, S.Jr. and Tobes, M.C. (1986) Morphologic and biochemical variability of tissue and cultured cells from human pheochromocytoma. J. Cell Physiol. **128**: 261-270.

10. Takiyyuddin, M.A., Cervenka, J.H., Sullivan, P.A., Pandian, M.R., Parmer, R.J., Barbosa, J.A. and O'Connor, D.T. (1990) Is physiologic sympathoadrenal catecholamine release exocytotic in humans. Circulation **81**: 185-195.

11. Peterkofsky, B. and Prather, W. (1976) Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. J. Cell Physiol. **90**: 61-70.

12. Menniti, F.S., Knoth, j. and Diliberto, E.J.Jr.. (1986) Role of ascorbic acid in dopamine β - hydroxylation. The endogenous enzyme cofactor and putative electron donor for cofactor. J. Biol. Chem. **261**: 16901-16908.

13. Jacob, H.S. and Lux, S.E. IV. (1968) Degradation of membrane phospholipids and thiols in peroxide hemolysis: studies in vitamin E deficiency. Blood **32:** 549-568.

14. Smolen, J.E. and Shohet, S.B. (1974) Permeability changes induced by peroxidation in liposomes prepared from human erythrocyte lipids. J. Lipid Res. **15**:273-280.