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Short Communications

Characterization of calcium oxalate crystals in woody plants by X-ray microarea diffractometry[1]

T. Taniguchi and H. Harada

Faculty of Education, Niigata University, Niigata 950-21 (Japan) and Faculty of Agriculture, Kyoto University, Kyoto 606 (Japan), 3 December 1986

Summary. Using X-ray microarea diffractometry, it was possible to characterize Ca oxalate crystals in situ. The Ca oxalate crystals in seven woody plant species were found to be present as the monohydrate ($CaC_2O_4 \cdot H_2O$).
Key words. Calcium oxalate crystals; X-ray diffractometry; tropical woody plants; *Populus nigra.*

In both plant and animal kingdoms calcium(Ca) oxalate crystals are recognized as biominerals, and many studies have been reported[2,3]. In animals, including man, urinary calculi are found, composed mainly of calcium oxalate, of calcium phosphate, and of mixtures of these[4]. In woody plants these crystals occur in ray and axial parenchyma cells[5,6]. Electron diffraction analysis in situ has been difficult, because strong bombardment of electrons changes the hydrate crystals into the corresponding anhydride.

The first investigation of the shape and distribution of crystals in woody plants was carried out under an optical microscope[7]. Since the crystals within a given taxon vary from species to species, they have been found to be a useful criterion in wood classification[8,9]. Until recently, characterization of these crystals in biological materials has been focused on studies of the chemical and optical properties of isolated crystals[10] by the use of X-ray diffraction[11] and infrared spectroscopy[12]. These analyses showed that Ca oxalate crystals in plants occur in two principal forms, monohydrate (whewellite) and dihydrate (weddellite). However, the results were sometimes contradictory and confusing, because the crystals were difficult to isolate and they contained various forms in one cluster. An in situ structural analysis of the crystals in woody plants has so far not been successful in allowing proper manipulation, and the hydrate compound usually decomposes into its anhydride form as a result of the electron bombardment involved in the electron diffraction technique. The object of this study was to establish a method of characterizing the crystals in situ by using X-ray diffractometry, which is now an established tool in investigating crystal structure nondestructively.

Materials and methods. Wood species were selected from six tropical woody plants which gave a wide range of crystal forms such as druses, styloids, prismatic crystals, needles, crystal sand, and raphides. From these species sections (60–100 μm) were prepared using a cryomicrotome cooled with dry ice at −45 °C. X-ray diffraction diagrams were obtained for the crystals by an X-ray microbeam, which was focused with a 30-μm diameter collimator set on a rotating anode X-ray diffractometer (model MDG-2193V, Rigaku Corporation, Japan).

For in vivo observation, the phloem tissues of Lombardy poplar (*Populus nigra* Linnaeus var. italica Duroi), which were cut from the inner bark of this specimen, were immediately dipped into isotonic aqueous 0.25 mol/l glucose solution, and were sectioned in the wet state by a sliding microtome. The section was set into an aluminum holder with a mica film window filled with the medium. Then an X-ray microbeam was focused on the crystal in situ in the phloem cells, and diffraction diagrams were obtained. Using the same sections on which X-ray diffraction analysis had been performed, we examined the location and the form of the crystal in situ by a scanning electron miroscopy.

Results and discussion. X-ray microarea diffraction photographs (fig. 1, A-E) of the crystals in situ in tropical woody plant cells showed a distinct, sharp point diffraction, but that of raphides showed a circularly arched diffraction spot (fig. 1, F). We interpreted these results as meaning that the styloid, prismatic crystal, needle, and crystal sand were composed of a single crystal, whereas the druses of the conglomerates and the raphides of the bundle consisted of single crystals. The d-spacings of the X-ray diffraction pattern of

Comparison of X-ray microarea diffraction data of druses (fig. 1, A) and that of Ca oxalate monohydrate listed in JCPDS files*

Observed d-spacing	d-values of $CaC_2O_4 \cdot H_2O$*
5.928	5.93
5.780	5.79
3.783	3.78
3.649	3.65
2.997	3.01
2.965	2.966
2.948	2.95
2.842	2.840
2.494	2.494
2.412	2.417
2.355	2.355
2.345	2.347
2.261	2.263
2.250	2.254
2.208	2.210
2.075	2.075
1.977	1.978
1.949	1.950
1.891	1.890

* Values of relative intensity more than the number of eight listed in Joint Committee on Powder Diffraction Standard File 20-231.

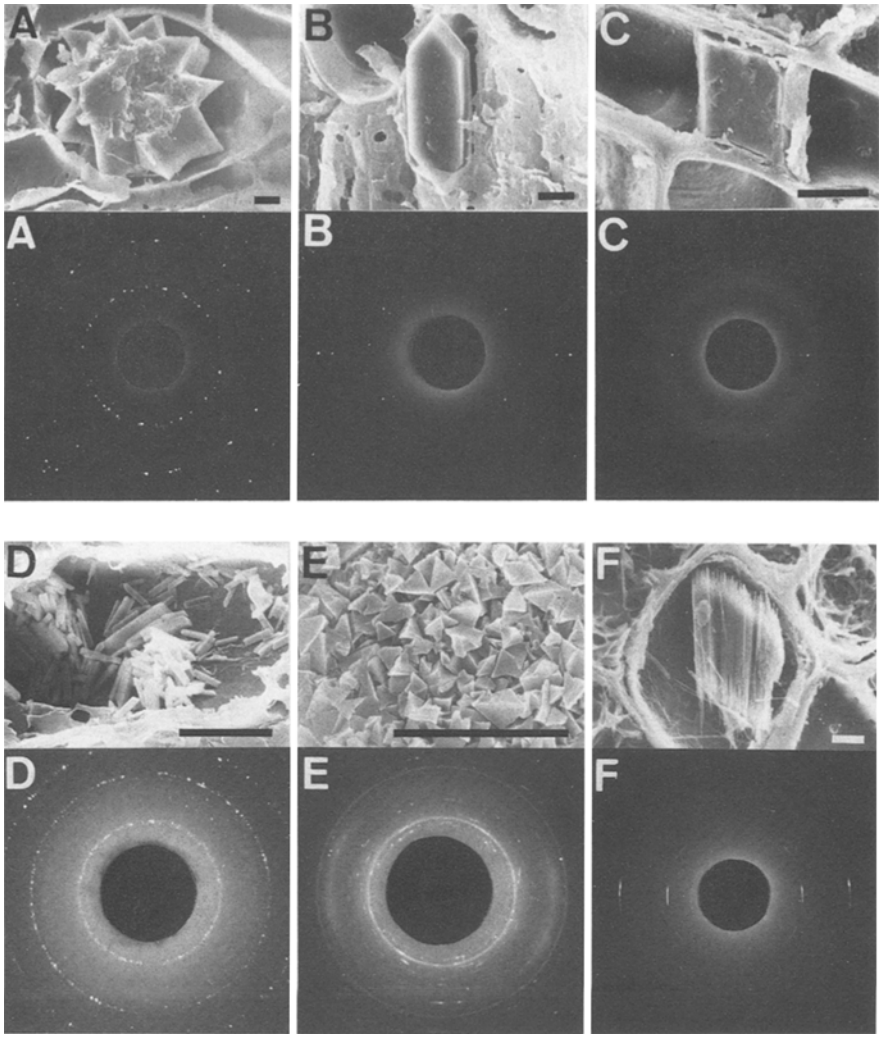

Figure 1. Scanning electron micrographs and X-ray diffraction photographs obtained for druses (A), styloid (B), prismatic crystal (C), needle (D), crystal sand (E), and raphides (F) in tropical woody plants cells. The X-ray wavelength was 0.154 nm, and the sample was oscillated around its vertical axis with an angle of ±30°. Diffraction pattern was obtained by a flat-film camera. *A, B,* and *C,* in axial parenchyma cells of *Terminalia solomonensis* Exell, *Terminalia calamansanai* Rolfe, and *Shorea sp.,* respectively; *D, E,* and *F,* in ray parenchyma cells of *Litsea timoriana* Span., *Cordia subcordata* Lam., and *Dillenia sp.,* respectively. Scale bar in micrograph, 10 μm.

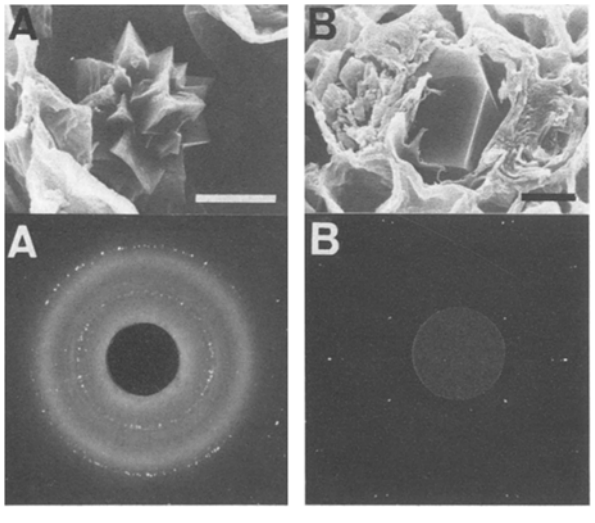

Figure 2. Scanning electron micrographs and X-ray diffraction photographs of druses (A) and prismatic crystal (B) in parenchyma cells of the phloem of Lombardy poplar (*Populus nigra* Linnaeus), in a wet state with 0.25 mol/l glucose solution. Scale bar in micrograph, 10 μm.

these crystals corresponded to the major known values for Ca oxalate monohydrate ($CaC_2O_4 \cdot H_2O$)[13] (table). It has been reported that the crystal shape is related to the hydration form of Ca oxalate[14]: the raphides from various plants have been identified with the monohydrate[12,15,16], whereas the druses and the prismatic crystals have been identified as being the monohydrate[17] or the dihydrate[16]. In the seven species in which we examined the crystals in situ, no hydrate form other than Ca oxalate monohydrate was present.

This microarea X-ray diffraction method is very useful for coming to an unequivocal conclusion, since even with small crystals in woody plant cells one can investigate the structure in situ. We applied the method to an intact living system of phloem cells from Lombardy poplar, which included various forms of crystal in the same species. X-ray microarea diffraction photographs of druses of the phloem cells in a wet state showed circularly dispersed diffraction spots (fig. 2, A), and those of prismatic crystals showed a point-diffraction pattern (fig. 2, B). These results suggest that both the druse form and the prismatic form in the phloem cells in vivo consist of Ca oxalate monohydrate, which formed a cluster of individual crystals upon crystallization.

All of the Ca oxalate deposits in the ray and axial parenchyma cells of xylem or phloem of the woody plants observed

here were found to be of the monohydrate type. We conclude that X-ray microarea diffractometry, as demonstrated here, is indeed a valuable method for examining the structure of biominerals in situ on a molecular level, and it can be expected to give a considerable impetus to structural analysis in the fields of biology and medicine.

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Microinjection of synthetic protein kinase inhibitor into single barnacle muscle fibers before and after cyclic AMP

E.E. Bittar and J. Nwoga[1,2]

Department of Physiology, University of Wisconsin, Madison (Wisconsin 53706, USA), 4 February 1987

Summary. Single muscle fibers from the barnacle *Balanus nubilus* have been used as a preparation to see if a synthetic 20-residue PKI (5–24)-peptide is able to block or reverse the stimulatory response of the ouabain-insensitive Na efflux to injected cAMP. The results obtained show that this peptide behaves as a powerful inhibitor of the cAMP-mediated response and is also able to partially reverse the sustained stimulation of the Na efflux observed in ouabain-poisoned fibers following the injection of subunit A of cholera toxin.
Key words. Synthetic PKI; cAMP; sodium efflux; barnacle muscle fibers.

Earlier work involving single muscle fibers from the barnacle, *Balanus nubilus* has shown that the Na efflux is stimulated by the microinjection of cyclic AMP[3] in a concentration as low as 10^{-6} M[4] and that pure protein kinase inhibitor (PKI) has the ability following its injection to interrupt or reverse the effect of cAMP[5], as well as that of pure catalytic subunit of cAMP-dependent protein kinase[6]. These observations are in keeping with the widely held view that PKI specifically inhibits the free catalytic subunit of cAMP-protein kinase, which is formed as the result of the dissociation of the holoenzyme by cAMP[7,8]. More recently, Scott and coworkers[9–11] have succeeded in defining the inhibitory domain of PKI and reported it to be represented by a 20-peptide residue. They also showed that potent inhibitory analogs contain at least 20–24 residues and that the 20-residue PKI (5–24)-peptide exhibits a Ki of ~ 9 nM. That is to say, it is as potent as native PKI. The purpose of this communication is to report that this particular peptide when microinjected into single barnacle muscle fibers behaves as a powerful inhibitor of the stimulatory response of the ouabain-insensitive Na efflux to injected cAMP.

Specimens of the barnacle *Balanus nubilus* were obtained from the Pacific Biomarine Laboratory, Inc. in Venice, CA, and kept in a 150-gallon Instant Ocean aquarium containing artificial seawater. The temperature of the aquarium water was maintained at about 12 °C throughout. Single fibers measuring 3–4 cm in length and 1–2 mm in diameter were isolated by dissection from the depressor muscle bundles and then cannulated. A 50–80-mg weight was attached to the tendon of the cannulated fiber, thereby keeping it in a vertical position while suspended in artificial seawater (ASW). The experiments were carried out with ASW having the following composition (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10 and pH 7.8. The microinjector used was of the type described by Bittar and Tallitsch[12]. The volume of test fluid injected into a fiber was about 0.4 µl. This is diluted by the myoplasm by a factor of roughly 100. $^{22}NaCl$ in aqueous solution was obtained from Amersham-Searle Corp., Arlington Heights, IL. The solution was dried down and then redissolved in distilled water, so that volumes of around 0.4 µl gave at least 750,000 counts per min. The effluent from the cannulated fiber loaded with radiosodium was collected every 5 min and the residual fiber activity was determined at the end of the experiment. A Beckman au-

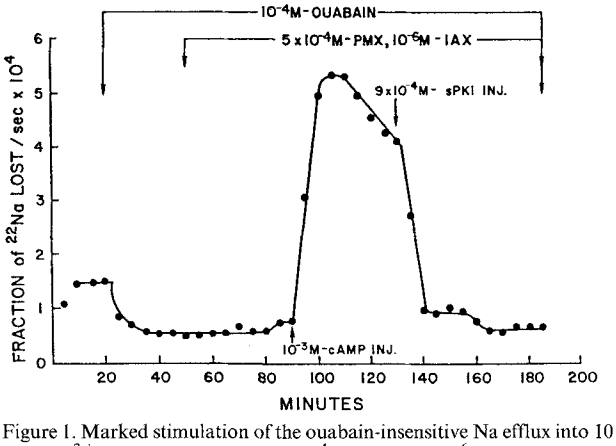

Figure 1. Marked stimulation of the ouabain-insensitive Na efflux into 10 mM-Mg^{2+}-ASW containing 5×10^{-4} M PMX & 10^{-6} M IAX by injecting 10^{-3} M cAMP and complete reversal of this response by injecting 9×10^{-4} M sPKI (rate constant for ^{22}Na efflux plot).