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Enhancement of fluorescence of pyrene-containing lipids by polar media, detergents and phospholipids

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Summary. The fluorescence intensities of a medium-chain fatty acid and of several amphiphilic lipids, each containing pyrene in covalent linkage, were enhanced considerably by: 1) Dissolving in mixtures of a polar solvent (e.g. methanol, ethanol, tetrahydrofuran or dimethylsulfoxide) and water; for each individual compound, a certain ratio of solvent to water provided maximal fluorescence intensity. 2) Incorporating into micelles of reduced Triton X-100; an excess of detergent was used so that, statistically, only one molecule of lipid resided in one micelle of the Triton X-100. 3) Incorporating into liposomes of egg phosphatidylcholine; maximal fluorescence was observed using a large excess of phosphatidylcholine. When related to the fluorescence intensities in chloroform/methanol (2:1, by vol.) or water, the enhancement of fluorescence in the above three systems was about 2–6-fold or up to 60-fold, respectively.
Key words. Fluorescent lipids; pyrene-labeled lipids; environment (effect on fluorescence).

Introduction

Pyrene, a polycyclic hydrocarbon, exhibits an intense fluorescence in the ultraviolet region[1]. It has been linked covalently to short, medium and long-chain fatty acids[2–5], and these subsequently bound to neutral[6–9], phospho[3, 10–14] and glycolipids[15]. Use of the pyrene fatty acids and their phospholipid derivatives to measure lateral diffusion, lipid exchange between different vesicles, translocation, phase separation phenomena and lipid-protein interactions has been reviewed[3]. In our previous studies, pyrene-linked lipids were used as substrates for lipolytic enzymes, e.g. sphingomyelinase and lipase[9, 13, 14]. The fluorescence intensities of the products were used as a measure of the activity of the respective enzymes. In other studies, pyrene fatty acids have been employed for following their transport across the plasma membrane of cultured cells[4, 16]. These acids, as well as several pyrene-containing lipids, have also been used for following continuously their insertion into liposomes[17] and uptake into cultured cells derived from normal individuals or patients affected with lipid storage diseases[14, 18, 19]. For quantitation, the products of enzymatic or cellular metabolic reactions were extracted with organic solvents, employing the procedures of Folch et al.[20] or Dole[21] and their fluorescence intensities recorded in mixtures of chloroform and methanol or in a heptane-rich phase, respectively.

While developing these procedures, we encountered systems in which the fluorescence intensities of the pyrene-lipids exceeded several-fold those recorded in the above-mentioned solvents. Using the latter could potentially increase the sensitivity of analysis in enzymatic or metabolic reaction studies. In this paper, three such systems are described for enhancing the fluorescence of pyrene derivatives of fatty acids and several amphiphilic lipids. For this purpose, the fatty acid or lipid was dissolved in mixtures of a polar solvent and water, or incorporated into micelles of a nonionic detergent (reduced Triton X-100) or liposomes of an unsaturated phospholipid (egg phosphatidylcholine). The latter two procedures are of special interest since they permit high-sensitivity spectrofluorometric measurement of the fluorescence intensities of lipids in aqueous media devoid of organic solvents, thereby making them especially useful for laboratories not fully versed in techniques of lipidology.

Materials and methods

Chemicals. 10-(1-pyrene)-decanoic acid (P10), 12-(1-pyrene)-dodecanoic acid (P12) and 1-pyrenesulfonyl chloride were purchased from Molecular Probes (Junction City, OR, USA). 12-(1-pyrenesulfonyl)-aminododecanoic acid (PSA12) was synthesized from 1-pyrenesulfonyl chloride and 12-aminododecanoic acid (Aldrich Chemical Corp., Milwaukee, WI, USA) following the procedure of Goldberg et al.[22] modified in that the 1-pyrenesulfonyl chloride was dissolved in dichloromethane. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride was obtained from Story Chemical Corp. (Muskegon, MI, USA); N,N'-dicyclohexylcarbodiimide and reduced Triton X-100 from Sigma (St. Louis, MO, USA); silica gel 60 H from Merck (Darmstadt, FRG); sodium taurocholate from Calbiochem (La Jolla, CA, USA) and sodium dodecylsulfate from Bio-Rad (Richmond, CA, USA). Sphingomyelin and sulfatide were purified from bovine brain[23, 24], glucosylceramide from the spleen of a patient with type I Gaucher disease[25] and phosphatidylcholine from egg yolk. All solvents were of analytical grade. Fluorescence measurements were recorded on a Perkin-Elmer LS-5 spectrofluorometer.

Synthesis of fluorescent derivatives of lipids. The following lipids were deacylated as previously described: sphingomyelin, by acid hydrolysis[23, 26]; glucosylceramide and sulfatide,

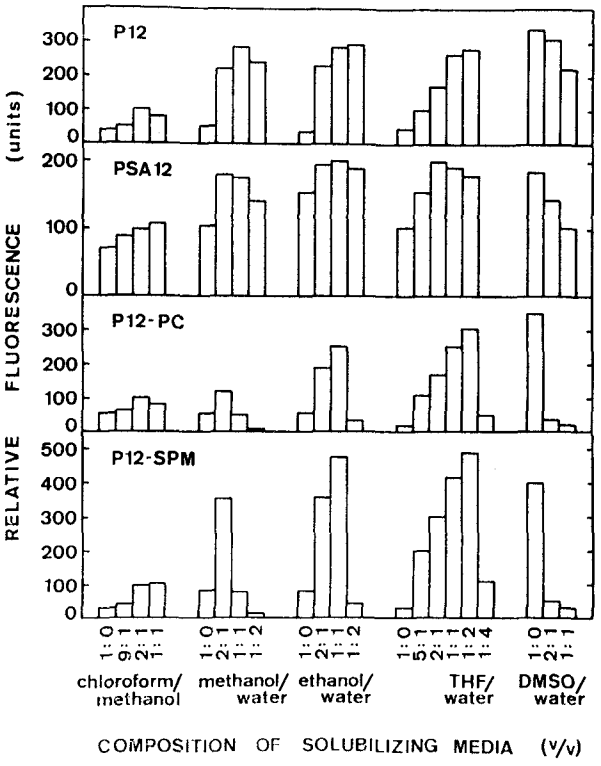

Figure 1. Effect of solvent mixtures on the fluorescence intensities of pyrene-containing lipids. Chloroform/methanol (2:1, by vol.) solutions containing 100 pmol, each of the respective lipids, were evaporated under nitrogen and 1 ml of solvent or solvent mixture added. The fluorescence intensities were recorded using excitation and emission wavelengths of 343 and 378 nm for the P12 derivatives, and 351 and 380 nm, respectively, for the PSA12 derivatives. All fluorescence values shown on the ordinate are related to the fluorescence intensity of solutions of each respective compound in chloroform/methanol (2:1, by vol.). In each case, the latter was assigned a value of 100 fluorescence units. The fluorescence intensities recorded for the solutions in chloroform/methanol (2:1, by vol.) were 37, 155, 53 and 44 units for P12, PSA12, P12-phosphatidylcholine (P12-PC) and P12-sphingomyelin (P12-SPM), respectively. Abbreviations of solvents are: THF, tetrahydrofuran; DMSO, dimethylsulfoxide.

by alkaline hydrolysis[24,27] and phosphatidylcholine by enzymatic hydrolysis[28]. Fluorescent derivatives of sphingomyelin were synthesized by condensing sphingosyl-phosphorylcholine with P10, P12 or PSA12 respectively, according to alternative procedures[23,29]. Fluorescent derivatives of ceramide were synthesized using similar procedures, starting from the appropriate pyrene fatty acid and sphingosine (obtained by acid hydrolysis of sphingomyelin). N-[12-(1-pyrene)-dodecanoyl]-sphingosyl-O-β-D-glucoside or P12-glucosyl-ceramide (P12-GC) was prepared according to Dinur et al.[25], egg phosphatidylcholine in which the unsaturated fatty acid in the β-position was replaced by P12 (P12-PC) and N-[12-(1-pyrene)-dodecanoyl]-sphingosyl-O-β-D-galactosyl-3-sulfate or P12-sulfatide (P12-CS) were synthesized by condensing the respective deacylated compounds with P12 in the presence of N,N'-dicyclohexyl-carbodiimide[28] [Dagan, A., and Gatt, S., unpublished data]. Each of these fluorescent derivatives of lipids was purified by preparative thin-layer chromatography and stored in the dark, at $-20\,^{\circ}C$, as a solution in chloroform/methanol (2:1, by vol.). Their concentration was estimated by determining the phosphorus content[30] or spectrophotometrically, assuming a value of 26,000 OD units/mole/cm (in chloroform/methanol, 2:1, by vol.) for the molar extinction coefficient of the pyrene-containing fatty acid.

Results

Figure 1 shows the fluorescence intensities of pyrene-containing fatty acids and phospholipids (0.1 μM) dissolved in mixtures of several polar solvents and water. The recorded fluorescence intensities in each solvent mixture are presented on the ordinate in values related to those observed in a mixture of chloroform/methanol (2:1, by vol.) which, for each compound, have been assigned a value of 100. The figure shows that, for each pyrene-lipid, a suitable solvent mixture could be selected which provided fluorescence intensities 3-5-fold greater than those observed using a chloroform/methanol solution. Among the 4 compounds used, pyrenesulfonylaminododecanoic acid had a relatively high fluorescence in chloroform/methanol and was affected to a lesser extent by the various solvent mixtures. Optical density measurements suggested the absence of any internal filter effect (data not shown).

The following experiments were aimed at obtaining high fluorescence of pyrene-containing lipids in aqueous media devoid of organic solvents. The table compares the fluorescence intensities in chloroform/methanol (2:1, by vol.), in water or in 0.1% reduced Triton X-100, of 0.1 μM each of dodecanoic acid, sphingomyelin and ceramide to which a pyrene or pyrenesulfonylamino group has been covalently linked. It should be emphasized (see 'Discussion') that, under these conditions, the Triton is present in a large excess relative to the lipid so that, statistically, a micelle of detergent contains only one molecule of lipid. As shown in the table, the fluorescence intensities of the various compounds in the aqueous, micellar dispersion of the Triton X-100 were 26–66 or 1.7–6.4-fold greater than the respective values observed in water or in chloroform/methanol (2:1, by vol.), respectively. PSA12 had a relatively high fluorescence in water so that the enhancement by the Triton was only 2.2-fold. Aqueous dispersions of the respective lipids in sodium tauchrocholate (9.3 mM) or sodium dodecylsulfate (17.4 mM) provided fluorescence intensities considerably lower than those observed in the presence of the reduced Triton X-100 (data not shown).

Considerable enhancement of the fluorescence intensities of pyrene-containing fatty acids or lipids could also be obtained by incorporating them into a liposomal dispersion of phosphatidylcholine. This is exemplified by the experiment of figure 2, in which P10-sphingomyelin has been incorporated into multilamellar liposomes of egg phosphatidylcholine. At a molar ratio of phosphatidylcholine to P10-sphingomyelin

Effect of Reduced Triton X-100 on the fluorescence intensities of aqueous dispersions of pyrene-containing lipids

Compound	Fluorescence (units)		
	Chloroform/ methanol (2:1, by vol.)	Water	Reduced Triton X-100 (0.1%)
P12	31	6	199
P12-sphingomyelin	35	3	198
P12-ceramide	34	5	204
PSA12	153	118	265
PSA12-sphingomyelin	154	9	312
PSA12-ceramide	207	15	389

Pyrene derivatives of fatty acids, sphingomyelins and ceramides (200 pmol) were dissolved in 2 ml of chloroform/methanol (2:1, by vol.) and their fluorescence was recorded. In the second set of test tubes, the solvent was evaporated under nitrogen and the residue dispersed in 2 ml of water by stirring on a vortex cyclomixer. In the third set, 2 mg of reduced Triton X-100 was added to the solution of the pyrene-lipid, the solvent was evaporated and the residue dispersed in 2 ml of water. The fluorescence intensities were determined using excitation and emission wavelengths of 343 and 378 nm, and 351 and 380 nm for P12 and PSA12 derivatives, respectively. All experiments were performed at least in triplicate.

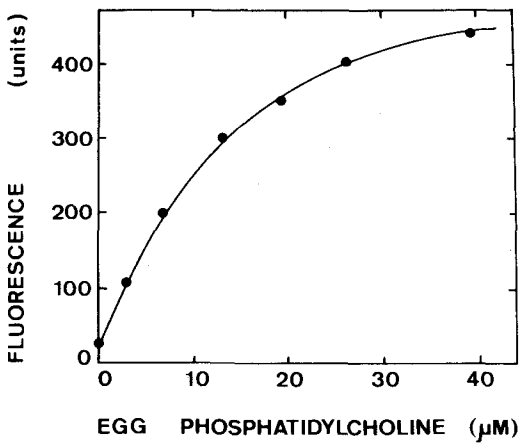

Figure 2. Effect of egg phosphatidylcholine on the fluorescence intensity of aqueous dispersions of P10-sphingomyelin. Chloroform/methanol (2:;1, by vol.) solutions of P10-sphingomyelin (containing 100 pmol) and of egg phosphatidylcholine were mixed and evaporated under nitrogen. 1 ml of water was added to each tube and, after thorough mixing on a vortex cyclomixer, the fluorescence intensity was determined using excitation and emission wavelengths of 343 and 378 nm, respectively.

of 400 (initial concentration of P10-sphingomyelin, 0.1 μM), the fluorescence intensity at 378 nm was enhanced more than 16-fold. Similar data were obtained when egg phosphatidylcholine was replaced by bovine brain sphingomyelin or a diunsaturated phosphatidylcholine, isolated from soybean phosphatides, or saturated phospholipids (distearoylphosphatidylcholine or dipalmitolyphosphatidylcholine) provided the experiment was performed above the phase transition temperature of the lipid (data not shown). Similar enhancement of the fluorescence of P12-sphingomyelin in water was also obtained when this compound was dispersed in small unilamellar vesicles of egg phosphatidylcholine using ultrasonic irradiation (data not shown).

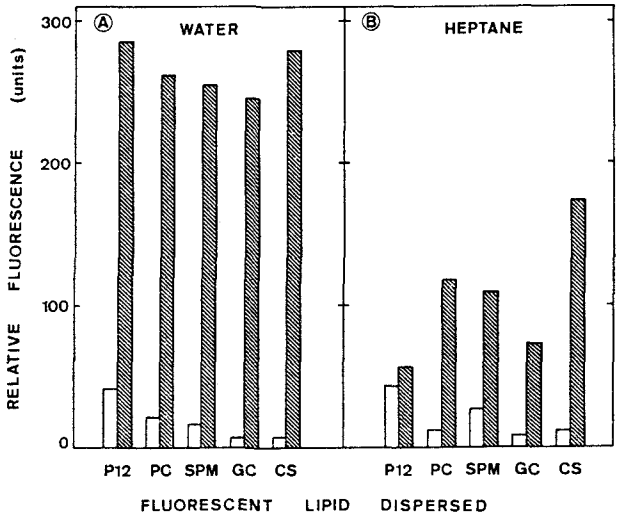

Figure 3. Effect of egg phosphatidylcholine on the fluorescence intensities of several fluorescent derivatives of lipids in water or in heptane. P12 derivatives of the respective lipids (200 pmol) were dissolved in chloroform/methanol (2:1, by vol.) and the solvent evaporated under nitrogen (open bars). In parallel, 50 nmol of egg phosphatidylcholine was mixed with each of the above lipids and the solvent similarly evaporated (hatched bars). 1 ml of water (A) or heptane (B) was added and the tubes were stirred, for 1 min on a vortex cyclomixer. The fluorescence intensities were recorded using excitation and emission wavelengths of 343 and 378 nm, respectively. All experiments were performed at least in duplicate. The values on the ordinate are related to the fluorescence intensities of chloroform/methanol (2:1, by vol.) solutions of 200 pmol of each lipid, which were each assigned a value of 100 units. For abbreviations of fluorescent lipids, see text.

Figure 3A presents values for enhancement, by egg phosphatidylcholine, of the fluorescence intensities of aqueous dispersions of P12 and several P12-containing lipids. All values are related to a value of 100 assigned to a solution of the corresponding lipid in chloroform/methanol (2:1, by vol.). Depending on the compound, the enhancement by phosphatidylcholine was about 2.5–3 times relative to chloroform/methanol solutions and about 7–35-fold relative to their dispersions in water.

Egg phosphatidylcholine could also be used for 'solubilizing' a pyrene-containing fatty acid and lipids in heptane, a hydrocarbon which normally does not dissolve polar lipids. As shown in figure 3B, the fluorescence intensities of the respective, mixed dispersions in heptane were only 1.3-fold greater for the fatty acid but 4–12-fold greater for the pyrene-containing complex lipids relative to their dispersions in heptane without phosphatidylcholine. A detailed analysis showed that under the experimental conditions of figure 3B, practically all the P12-sphingomyelin had indeed been dispersed in the heptane while, in the absence of egg phosphatidylcholine, most of the sphingomyelin remained attached to the walls of the tube.

Discussion

Fluorescence emission of a fluorophore can be strongly influenced by the medium in which this compound is dissolved. For example, it has long been known that the fluorescence quantum yield of aromatic compounds, e.g. pyrene, is solvent dependent[31]. Another interesting example pertains to pyrene-fatty acids in aqueous media. Thus, pyrene-dodecanoic acid in water exhibits a rather low fluorescence emission intensity relative to that observed in an organic solution. But, when complexed with albumin in an aqueous medium, the fluorescence increased many-fold[17]. This is probably due to binding of the pyrene-moiety of the fatty acid to a hydrophobic region of the albumin. Another reason for variation of the fluorescence intensity is related to the excimer formation property of pyrene derivatives. Thus, when a molecular solution of a pyrene-containing compound is irradiated at the excitation peak of 343 nm, fluorescence emission occurs at 378 nm (monomer fluorescence). But, in an aggregated state, the excited pyrene molecule can interact with a non-excited pyrene molecule to form an excimer which fluoresces at about 475 nm (excimer fluorescence). The ratio of excimer to monomer fluorescence intensities is proportional to the pyrene derivative concentration[32]. The excimeric emission will predominate in aqueous dispersions of most lipids, either those forming micelles (e.g. fatty acids, sulfatides or gangliosides), or those forming liposomes (e.g. glycerophosphatides and sphingomyelins) or undefined dispersions (e.g. cerebrosides, neutral glycerides and cholesteryl fatty acyl esters). Breaking up such dispersions into monomolecular solutions or, alternatively incorporating these lipid molecules into systems which decrease the statistical chance that two adjacent pyrene molecules will interact, will result in a decrease in the excimeric and an increase in the monomeric emission intensities. This can be achieved by dissolving pyrene-lipids in organic solvents where they form molecular solutions or attaching them to proteins such as albumin, selecting a ratio which will have one pyrene-lipid bound to one molecule of albumin. A similar increase in monomeric emission was also observed when micelles of pyrene-fatty acids released monomers which were incorporated into liposomal or cellular membranes. In fact, the appearance of an intense emission peak at 378 nm permitted continuous spectrofluorometric monitoring of phenomena such as incorporation[17].

In this study, we aimed at enhancing the fluorescence intensity of pyrene-containing fatty acids and amphiphilic lipids, especially in aqueous media. This was done in three systems. In the first, mixtures of polar solvents with increasing proportions of water were used for dissolving pyrene-fatty acids or phospholipids. For each solvent (i.e. methanol, ethanol, tetrahydrofuran or dimethylsulfoxide), somewhat different proportions of solvent and water provided the maximal fluorescence intensity, which was 2–5-fold greater than that of a chloroform/methanol (2:1, by vol.) solution of the same lipid.

In the second system used for enhancing the fluorescence intensities, the pyrene-fatty acids or lipids were incorporated into micelles of Triton X-100. This non-ionic detergent has a benzene ring which imparts to it a rather high background fluorescence. Therefore, a modified detergent in which the benzene ring had been reduced to form a cyclohexyl residue was used. This reduced Triton X-100, which is commercially available, is almost devoid of fluorescence. Excess detergent was used, so that, statistically, only one molecule of pyrene-lipid resided in one micelle of the non-ionic detergent. To exemplify, in the experiment of the table, 0.1 nmol of lipid was mixed with 1 ml of 0.1% Triton. This corresponds to about 1.5 mM detergent and, subtracting a critical micellar concentration of about 0.25 mM [33], the concentration of micellar detergent is 1.25 mM. Assuming that each micelle is composed of 134 molecules of detergent[34], the final concentration of the micelles will be close to 10 $nmol \cdot ml^{-1}$ or, about 100 micelles per each molecule of pyrene-fatty acid or pyrene-lipid. At this ratio, all the lipid will be incorporated into the detergent micelles and, because of the extremely low chance for two pyrene molecules residing in the same micelle, the entire fluorescence emission will appear at the monomeric peak. Furthermore, the pyrene moiety of the lipid will be located in the hydrophobic, isooctylcyclohexyl moiety of the micelle providing a high fluorescence intensity. Indeed, relative to the fluorescence in water, up to 66-fold greater values were measured in the presence of the reduced Triton X-100. Relative to the fluorescence in chloroform/methanol (2:1, by vol.), up to 6-fold greater intensities were recorded.

Not less striking are the data in which the pyrene complex lipids have been incorporated into multilamellar liposomes or small unilamellar vesicles of egg phosphatidylcholine. As shown in figures 2 and 3, the fluorescence intensities, at 378 nm, of the various lipids, relative to those observed in water, were up to 7-35-fold greater and, relative to those measured in chloroform/methanol (2:1, by vol.), about 3-fold higher. These observations are in good agreement with recently published results[35] on the effect of lipid bilayers on the fluorescence intensity of pyrene or pyrene derivatives (not pyrene complex lipids). The use of egg phosphatidylcholine also permitted the 'solubilizing' of pyrene-lipids in heptane. This might be a consequence of the formation of inverted mixed micelles in the hydrocarbon, in which the apolar moieties of phosphatidyl choline and pyrene-lipid are exposed to the solvent and the phosphocholine and polar portion of the fluorescent lipid (e.g. galactosylsulfate of the sulfatide) are in the interior of the micelle.

It is worth mentioning that either in the Triton X-100 or phosphatidylcholine dispersions, all the pyrene-lipids tested showed an emission only at the monomeric peak (i.e. 378 or 380 nm for P12 and PSA12 derivatives, respectively). The excimeric emission peak was absent in both systems.

The data reported in this paper emphasize the importance of adopting, for each fluorescent lipid, an optimal solvent mixture or hydrophobic environment which will permit optimal expression of its fluorescence emission. Using an unsuitable environment might decrease the fluorescence intensity up to more than 50-fold or even quench it completely. This was exemplified in another study[17] in which the fluorescence of a pyrene fatty acid-albumin complex was quenched by linking, covalently, to the latter protein, trinitrophenyl groups which absorbed the photons emitted from the excited pyrene molecule.

Use of micelles of the non-ionic detergent Triton X-100 or liposomes of egg phosphatidylcholine provided high fluorescence intensities of pyrene-fatty acids or lipids in aqueous media devoid of organic solvents. This might be especially useful for laboratories which are not well adapted for studies or analyses of lipids using organic solvents.

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Short Communications

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

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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.