Modulated Uric Acid Crystal Growth in the Presence of Acridine Dyes

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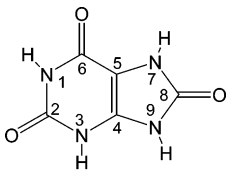
Single crystals of anhydrous uric acid (**UA**) and uric acid dihydrate (**UAD**) were grown from supersaturated aqueous solutions containing the fluorescent dye acriflavine neutral (**AN**), a two-component commercial mixture of proflavine (**P**), and its 10-methylated analogue acriflavine (**A**). During **UA** crystal growth at 37 °C, small quantities of **AN**, **P**, and **A** dye probes are found to be selectively included in {001} and {201} growth sectors. Increasing concentrations of dye in solution show a general retardation in the growth of (121) faces, resulting in an overall change from a rectangular to a prismatic habit. For **UAD** growth at 25 °C, **AN**, **P**, and **A** inclusion does not appear to favor any particular growth sector, and no discernible habit changes are observed under even the most concentrated dye solutions examined. The average orientation of dye molecules trapped in the **UA** and **UAD** matrixes was determined from polarization data obtained from a home-built microspectrometer. Dye-surface recognition events can be discerned on the basis of the calculated dye orientation. The difference in the interlayer distance between uric acid layers in **UA** and **UAD** and the resultant surface perturbations caused by dye recognition and inclusion can be used to rationalize the observed habit modification in the anhydrous form.

Introduction

Uric acid is a normal product of protein metabolism, but errors in its regulation can lead to elevated concentrations and its subsequent crystallization in vivo. Under low pH conditions, supersaturated uric acid solutions can yield either neutral uric acid (**UA**) or uric acid dihydrate (**UAD**) crystals, both of which have been found in human kidney stone deposits. Precipitation of monosodium urate crystals, the typical clinical symptom of gout,[1] occurs from supersaturated solutions with pHs higher than uric acid's pK_a of 5.4.[2]



Uric Acid

Synthetic crystals of **UA** (space group $P2_1/a$ and unit cell dimensions a = 14.464(3) Å, b = 7.403(2) Å, c = 6.208(1) Å, and β = 65.10(5)°)[3] can be reliably grown from supersaturated solutions at 37 °C. **UA** adopts a layered motif, with adjacent layers spaced 6.56 Å apart along a^* (Figure 1). Each layer consists of parallel ribbons of uric acid molecules hydrogen-bonded head-to-head and tail-to-tail, with the ribbon plane perpendicular to the $b-c$ plane. There is no hydrogen-bonding between ribbons within a layer, although ribbons in adjacent layers are hydrogen-bonded and offset by ~ 62°. **UA** crystallizes as clear rectangular plates, with large (100) faces bounded by (210), (201), (001), and sometimes very small (121) faces.[4]

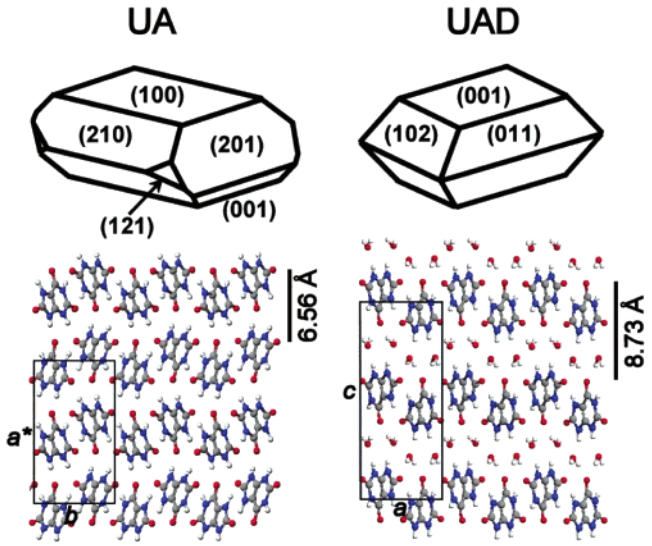


Figure 1. Crystal habit and structure of pure **UA** (left) and **UAD** (right).

UAD single crystals (space group $P2_1/c$ with a = 7.237(3) Å, b = 6.363(4) Å, c = 17.449(11) Å, and β =

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(1) Howell, R. R.; Evans, E. D.; Seegmiller, J. E. *Arth. Rheum.* **1963**, *6*, 91−103.
(2) Mandel, N. S.; Mandel, G. S. *J. Am. Chem. Soc.* **1976**, *98*, 2319−2323.
(3) Ringertz, H. *Acta Crystallogr.* **1966**, *20*, 397−403.
(4) Rinaudo, C.; Boistelle, R. *J. Cryst. Growth* **1980**, *49*, 569−579.

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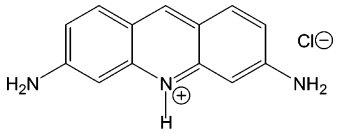
90.51(1)°)[5] can be grown from supersaturated solution at 24 °C, though most crystals are highly disordered and/or twinned. Crystals of **UAD** slowly dehydrate over time to yield polycrystalline **UA**.[6] The layer structure of **UAD** is very similar to that of **UA**, with the addition of hydrogen-bonded water molecules between adjacent uric acid layers increasing the overall layer spacing to 8.73 Å. **UAD** deposits as clear rectangular plates, with large (001) faces bounded by (011) and (102), and infrequently (210).[4]

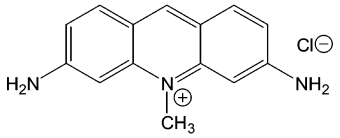
Although synthetic uric acid crystals are colorless and have well-defined shapes, those formed in vivo are invariably colored and adopt different habits, indicating the inclusion of impurities. Studies on uric acid crystallization in the presence of impurities date back to the 1930s, when Gaubert[7] (and later Kleeberg[8] in the 1970s) first grew uric acid in the presence of assorted synthetic and natural dyes. Though these studies offered little insight into a rational molecular-level understanding of the recognition events between the dyes and crystal surfaces, more recent work by Kahr[9] on other crystal hosts has shown that dye probes can be used as a very effective tool for studying crystal growth phenomena.

In reproducing some of the early uric acid studies, we discovered that dye inclusion in **UA** and **UAD** matrixes could occur in some cases with high specificity. We recently reported[10] our finding that methylene blue is selectively included in {001} and {201} growth sectors of **UA** crystals, whereas **UAD** grown under similar dye concentrations resulted in nonspecific dye inclusions throughout the entire crystal matrix. Interestingly, growth from even the most concentrated methylene blue solutions yielded **UA** and **UAD** crystals with rectangular habits identical to pure synthetic uric acid crystals.

The present study describes uric acid crystal growth in the presence of the two-component fluorescent dye acriflavine neutral (**AN**) and each of its separate components. Acriflavine neutral is sold commercially as an approximately 8:1[11] mixture of proflavine (**P**), 3,6-diaminoacridine, and its methylated analogue acriflavine (**A**), 3,6-diamino-10-methyl-acridinium hydrochloride. Acridines are known to interact strongly with nucleic acids in solution,[12] making them potentially good modulators of uric acid (a purine) crystal growth. Browning and Gilmour[13] had also shown, as early as 1913, that some of the acridine dyes (e.g., 9-aminoacridine, proflavine, and acriflavine) exhibit strong antibacterial properties, making them potentially viable therapeutics. The present study focuses on proflavine and acriflavine dyes which have a solution $Abs_{max} \sim 450$ nm. 9-Aminoacridine was not included in the present investigation because its absorbance occurs in the UV region ($Abs_{max} \sim 358$ nm), beyond the range of our custom-built visible micro-spectrophotometer.



Proflavine (P)



Acriflavine (A)

Experimental Section

Materials. Water was purified by passage through two Barnstead deionizing cartridges followed by distillation. Uric acid (Aldrich, 99+%), acriflavine neutral (Aldrich, CAS 8048-52-0), and 3,6-diaminoacridine hydrochloride (Aldrich, CAS 952-23-8) were used as received. 3,6-Diamino-10-methylacridinium chloride (CAS 86-40-8) was isolated from acriflavine neutral solution according to the method of Gailliot.[14] The separation was accomplished by adding a suspension of freshly prepared silver oxide to the acriflavine neutral solution. The precipitate was subsequently removed by vacuum filtration, and the filtrate was neutralized with hydrochloric acid and concentrated. Crystals of pure **A** form upon cooling and are recrystallized from water and methanol. ^{1}H NMR (Varian 300 MHz, DMSO-d_6) δ: 8.74 (s, 1H), 7.86 (d, 2H), 7.41 (s, 4H), 6.99 (d, 2H), 6.86 (s, 2H), 3.94 (s, 3H).

Crystal Growth. Supersaturated solutions were prepared by dissolving 15−20 mg of uric acid in 100 mL of boiling deionized water (0.9−1.2 mM). Pure uric acid dihydrate (**UAD**) and uric acid (**UA**) single crystals grew upon standing at room temperature (24 ± 1 °C) and in a water bath (37 ± 0.1 °C), respectively. The pH of unbuffered uric acid solutions is typically 4−5. Methods to identify **UA** and **UAD** matrixes by conoscopic light interference patterns have been described previously.[10]

Crystals were also grown in the presence of **AN**, **P**, and **A** dye probes. Stock dye solutions were prepared by dissolving 100 mg of either **AN**, **P**, or **A** in 100 mL of deionized water. Dye solution was added to the supersaturated uric acid solutions to yield a final dye concentration of 30−240 μM (~4−40:1 uric acid/dye). The resulting solutions were maintained at 24 or 37 °C. Because acridine is a photosensitive dye, all crystal growth occurs in either the dark or in amber vials. After 1−7 days, small (200−300 μm) plate-shaped single crystals with dye inclusions (**UA**−**AN**, **UA**−**P,** **UA**−**A**, **UAD**−**AN**, **UAD**−**P**, and **UAD**−**A**) were formed.

Optical Microscopy and Spectrophotometry. All crystal images were obtained using an Olympus BX50 polarizing microscope. Visible absorption spectroscopy of dye-inclusion crystals was achieved by interfacing the microscope with an Ocean Optics PC2000-ISA spectrometer. Absorption intensities between 400 and 750 nm could be obtained on crystal areas as small as 10−15 μm in diameter using the highest power objective (50×).[10] These dimensions are well-suited for dyed uric acid crystals, which typically reach only ~30 μm in their smallest dimension. Fluorescence microscopy imaging was accomplished by adding excitation (Schott B370) and emission (Schott OG530) filters.

Dipole Calculations. Visible transition dipoles for proflavine and acriflavine were calculated using the ZINDO module of CAChe V5.0 software (Fujitsu, Beaverton, OR).

(5) Parkin, S.; Hope, H. *Acta Crystallogr.* **1998**, *B54*, 339−344.
(6) Boistelle, R.; Rinaudo, C. *J. Cryst. Growth* **1981**, *53*, 1−9.
(7) Gaubert, P. *Comptes Rendus* **1936**, *202*, 1192−1194.
(8) Kleeberg, J.; Warski, E.; Shalitin, J. In *Purine Metabolism in Man: Biochemistry and Pharmacology of Uric Acid Metabolism*; Sperling, O., DeVries, A., Wyngaarden, J. B., Eds.; Plenum Press: New York, 1974; pp 821−833.
(9) Kahr, B.; Gurney, R. W. *Chem. Rev.* **2001**, *101*, 893−951.
(10) Sours, R. E.; Fink, D. A.; Swift, J. A. *J. Am. Chem. Soc.* **2002**, *124*, 8630−8636.
(11) Green, F. J. *The Sigma-Aldrich Handbook of Stains, Dyes and Indicators*; Aldrich Chemical Co: Milwaukee, WI, 1990.
(12) Acheson, R. M. *Acridines*, 2nd ed.; Interscience Publishers: New York, 1973; Vol. 9.
(13) Browning, C. G.; Gilmour, W. *J. Pathol. Bacteriol.* **1913**, *24*, 127.
(14) Gailliot, M. P. *Bull. Soc. Chim. Fr.* **1934**, *1*, 796−806.

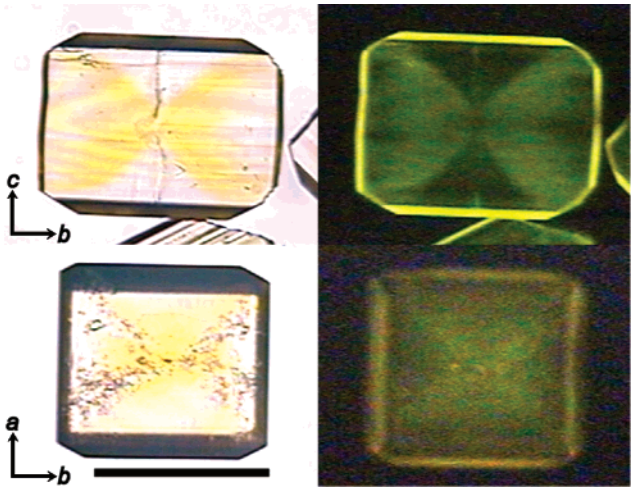


Figure 2. Polarized photomicrograph and fluorescence images of dye inclusion crystals. (top) A typical **UA**−**AN** inclusion crystal grown at 37 °C showing dye inclusion in {001} and {201} growth sectors. (bottom) A typical **UAD**−**AN** inclusion crystal grown at 24 °C. Scale bar = 100 μm. All crystals were grown in the dark.

Results and Discussion

Dye Inclusion Crystals. Dyes are excellent probes for crystal growth studies because inclusion of even small amounts in a crystalline host matrix can be detected. We grew crystals of uric acid from supersaturated aqueous solutions containing **AN**, **P**, and **A** concentrations ranging from 30 to 240 μM. **UA** crystals grown at 37 °C from solutions containing **AN** exhibited yellow dye inclusions (Figure 2). At lower dye concentration, the yellow inclusion was confined to an hourglass consisting of the {001} and {201} growth sectors. However, as the solution dye concentration increased, the yellow color became more pronounced and some dye appeared to be included throughout the crystal matrix. Though the dye inclusion pattern in many crystals is readily discerned by polarized microscopy, fluorescence microscopy was often more helpful at detecting the yellow-green dye fluorescence, especially under the lowest solution dye concentrations examined.

Interestingly, the **UA**−**AN** crystals also showed an obvious change in their macroscopic habit compared to that of unstained **UA** crystals. As the concentration of dye in the growth solution increased, the size of the {121} faces of the resulting crystals increased. Increasing growth retardation along [121] yields crystals with a predictable array of habits ranging from rectangular (no dye) to pseudohexagonal to prismatic (Figure 3).

The average concentration of dye included in uric acid crystals grown from solutions with varying dye concentrations (130−230 μM) was determined by absorbance measurements obtained on batches of dissolved **UA**−**AN** crystals. Over the solution concentration range examined, the number of matrix-occluded **AN** molecules increases by an order of magnitude: from 4 to 15 dye molecules per 10^4 molecules of uric acid.

UAD crystals grown from solutions containing similar dye concentrations (130−230 μM) yielded slightly different results. The amount of included dye was very consistent (6−7 **AN** molecules per 10^4 uric acid molecules) and was not affected by increasing solution concentrations. Dye inclusion in **UAD** does not, in general, appear to be specific for particular growth sectors, although at the highest dye concentrations some selectivity for {210} or {011} appears to be a possibility (Figure 2). When viewed under linearly polarized light, the yellow pattern is most intense when the incident polarization is parallel to the *b* axis of the crystal. This suggests that the dye molecules included in the crystal may be preferentially oriented, even though they lack selectivity for specific growth sectors. Unlike **UA**−**AN**, the **UAD**−**AN** crystals showed no change in their macroscopic habit or relative size compared to unstained **UAD** crystals, even at the highest solution dye concentrations examined.

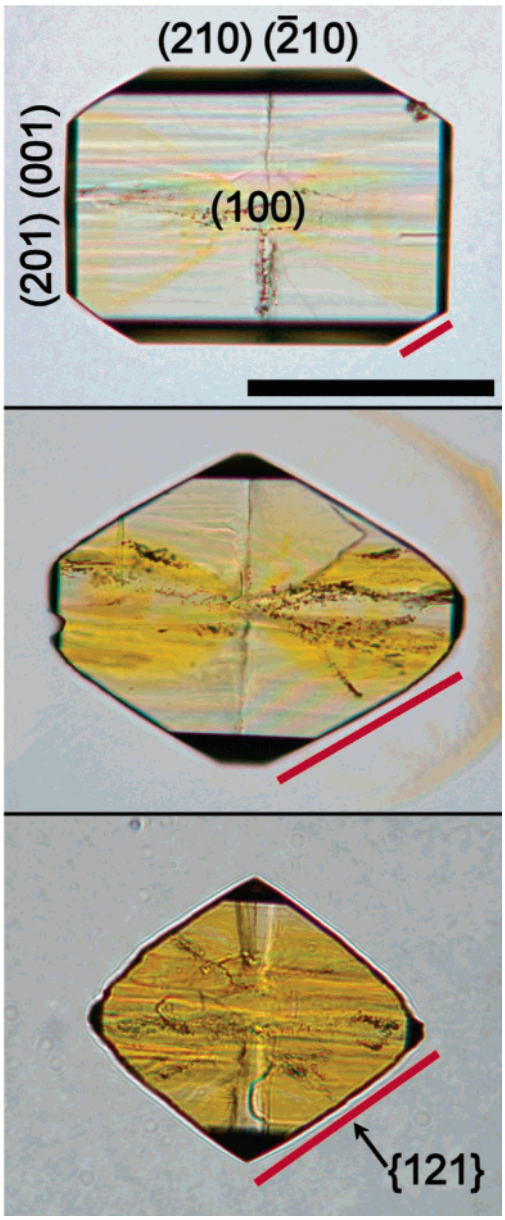


Figure 3. Polarized photomicrographs of **UA**−**AN** grown from solutions containing different dye concentrations. Increasing concentrations of acriflavine neutral in the uric acid growth solution led to habit modification by retardation of **UA** {121} faces. Dye concentrations: 130 (top), 165 (middle), and 230 μM (bottom). Scale bar = 100 μm.

Because **AN** is a two component dye, we were interested to see whether there was a preference for inclusion

of either the acriflavine or proflavine components in the uric acid matrixes. Growth of both **UA** and **UAD** in the presence of pure acriflavine (**A**) yielded **UA**−**A** and **UAD**−**A** dye-inclusion crystals, which were identical in all respects to analogous **UA**−**AN** and **UAD**−**AN**.

UAD crystals grown in the presence of proflavine (**P**) appeared essentially identical to **UAD**−**AN** and **UAD**−**A**. However, surprising difficulty was encountered in growing **UA** single crystals in the presence of **P**, even at the lowest solution dye concentrations examined. This qualitative observation suggests that proflavine may be a more efficient growth inhibitor for anhydrous uric acid than its 10-methyl analogue, though at present we are unable to provide a rational explanation to account for this behavior.

Linear Dichroism. Although electronic spectroscopy inherently averages orientational sub-populations (different orientations in distinct environments or aggregates), and can give rise to false calculated orientations, it is also a powerful tool for discerning the orientation of molecular species trapped in an ordered matrix. Visible absorption spectra for all dye-inclusion crystals were collected on our custom-built microspectrophotometer at two orthogonal polarization directions for each of the three orthogonal viewing directions. The small size and brittleness of the crystals prohibited cutting and polishing faces perpendicular to the *b* and *c* (**UA**) or *a* and *b* (**UAD**) axes, so measurements in those directions were made on the naturally angled faces.

The absorption spectra of **UA**−**AN** and **UAD**−**AN** are provided in Figures 4 and 5. Integrated intensities were calculated from a best fit of three Gaussians to baseline-corrected absorption spectra. Because the birefringence of **UA** and **UAD** ($B = 0.31$ and 0.22) is too large to ignore, Vuks' method[15] was used to correct the measured absorption intensities for matrix refractive index differences. No additional correction was applied to account for any minor reflection losses from the crystal surface.

Examination of the visible absorption spectra for solutions of **AN**, **A**, and **P** over the concentration range used for crystal growth provided no evidence for dye aggregation under these conditions. Furthermore, the absorbance maximum and intensity for each does not change appreciably over a pH range of 2−10. In dilute aqueous solutions, the maximum absorbances of **A** and **P** are 453 and 444 nm, respectively. Supersaturated uric acid solutions containing **AN**, **A**, and **P** exhibit red-shifts of 1−2 nm in their maximum visible absorbance.

Dyes trapped in organic crystal matrixes typically exhibit a larger red-shift in their visible absorption spectra. We found that the absorption maxima for **AN** in **UA**−**AN** and **UAD**−**AN** single crystals were 477 and 478 nm, respectively, and independent of the direction of incident polarization. The absorbance maxima of **UA**−**A** (481 nm), **UA**−**P** (475 nm), **UAD**−**A** (481 nm), and **UAD**−**P** (478 nm) also remained constant throughout all incident polarization directions. For all dye-inclusion crystals, peak broadening was observed when looking down edge faces, although the variation in peak wavelength observed was <5 nm. Crystals containing proflavine also had a small peak or shoulder at 601 nm (**UA**−**P**) or 593 nm (**UAD**−**P**).

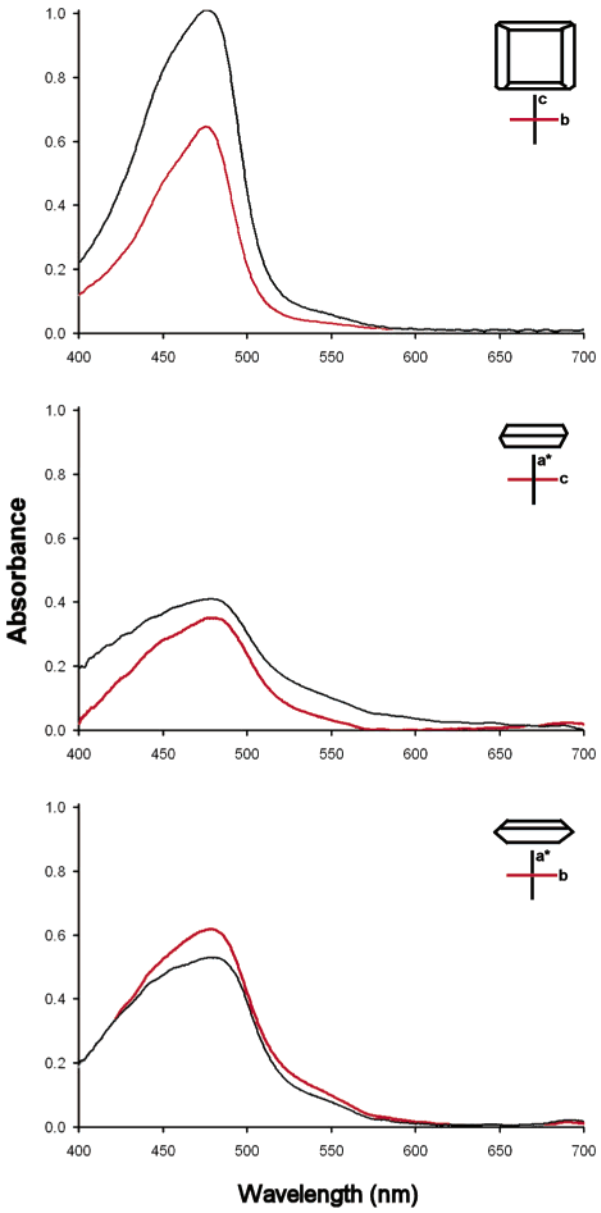


Figure 4. Baseline-corrected absorption spectra for **UA**−**AN** crystal with polarized light incident along the *a** axis (top), the *b* axis (middle), and the *c* axis (bottom). Labeled axes below crystal figures indicate the orientation of the incident polarization with respect to the crystal.

Both acriflavine and proflavine are cationic at the pHs under which uric acid crystal growth occurs.[16] Therefore, in all dye-inclusion crystals, an equal number of anions must also be included to maintain charge balance throughout the matrix. Obvious potential anions in solution include chloride (both dyes are chloride salts), hydroxide, and uric acid itself, which exists as urate in about 12% abundance at pH 4.5.

Dye Orientation. For both proflavine and acriflavine, the visible transition dipole lies along the long axis of the molecule. The calculated transition dipole and the linear dichroism measurements were used to construct molecular models for the dye orientation (Figure 6). In both **UA** and **UAD** matrixes, the dye orientation was found to coincide with the uric acid ribbon direction. We

(15) Vuks, M. F. *Opt. Spectrosc.* **1966**, *20*, 361−364.

(16) Albert, A. *The Acridines*, 2nd ed.; Edward Arnold Publishers Ltd: London, 1966.

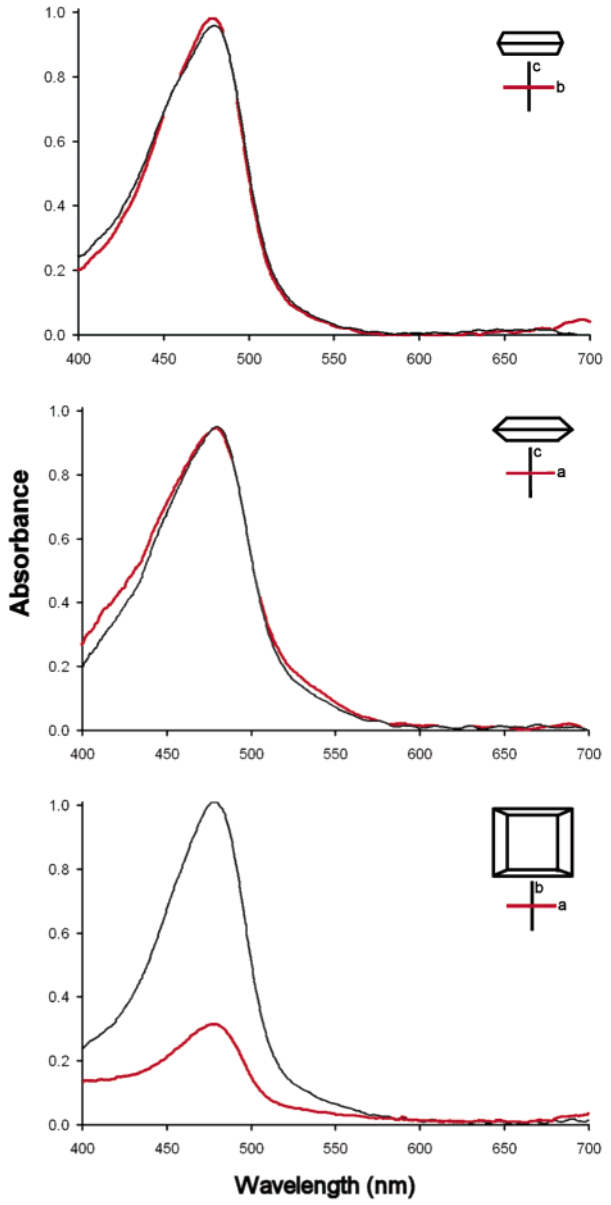


Figure 5. Baseline-corrected absorption spectra for **UAD**−**AN** crystal with polarized light incident along the *a* axis (top), the *b* axis (middle), and the *c* axis (bottom). Labeled axes below crystal figures indicate the orientation of the incident polarization with respect to the crystal.

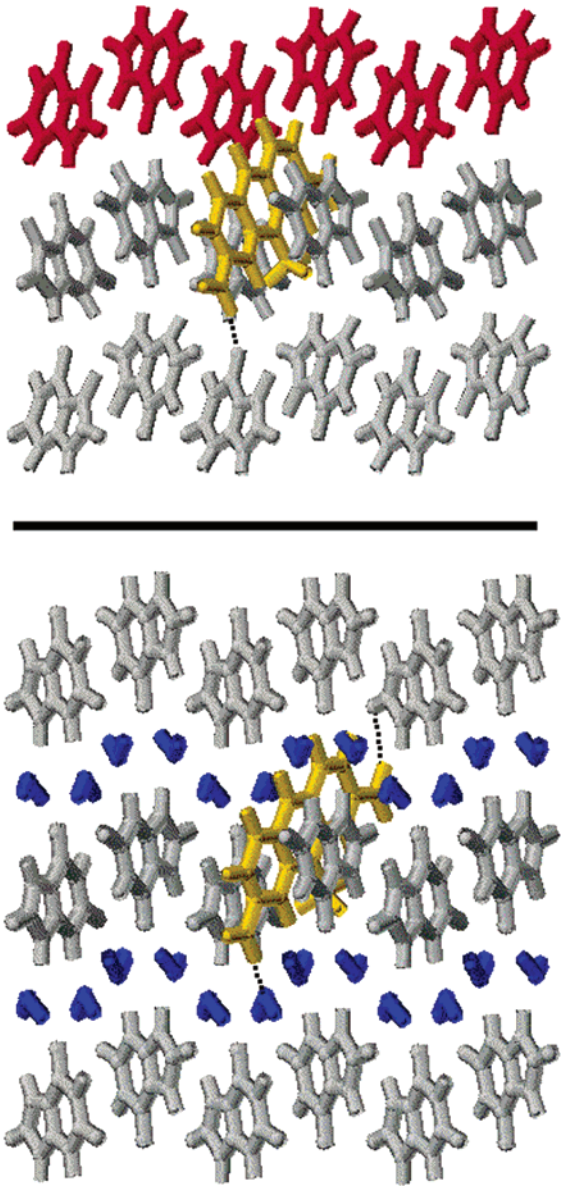


Figure 6. Proposed orientation of acriflavine dye in (top) **UA** and (bottom) **UAD** matrixes based on 3-D polarization data. When acriflavine substitutes for a uric acid molecule, it appears to "fit" within a single 8.73 Å layer of **UAD** more easily than in **UA**, which has a smaller 6.56 Å interlayer distance. Possible hydrogen bonds are indicated by dashed black lines. The protrusion of acriflavine on the surfaces of **UA** may likely alter growth of the overlying (red) layer, leading to larger (121) faces (acriflavine, yellow; uric acid, gray or red; water, blue).

assumed a face-to-face stacking interaction between uric acid and dye molecules in each crystal lattice, which limits the rotational orientation about the dye's long axis to two possibilities.

The translational position of the dye within the crystal matrix was inferred such that the dye is confined (as near as possible) to a single uric acid layer, based on steric considerations. This configuration also allows for optimal hydrogen bonding between the dye's amino hydrogen and either a carbonyl on the underlying uric acid layer (in **UA**) or a water molecule (in **UAD**). In Figure 6, the optimized H-bond distances and geometries depicted for acriflavine (yellow)−uric acid (gray) binding in **UA** matrixes affords an O···H−N angle of 161° (O···N = 2.7 Å). In comparison, the normal uric acid−uric acid hydrogen bond distance for that site is 2.73 Å (O···N) with an O···H−N angle of 159°.[3]

At this optimized geometry, the other end of the acridine protrudes slightly above the top surface of the uric acid molecules that make up the dye-included layer. Steric considerations dictate that at least two of the uric acid molecules in the next adjacent layer (red) will not be able to occupy their normal positions or orientations. The presentation of the dye's other amine (H-bond donor) on the surface may additionally confuse the orientation of uric acid molecules in the next uric acid layer during growth. Our experience with growth in the presence of other synthetic dyes bearing free amines revealed qualitatively similar habit changes. We believe these steric effects are the likely origin of the retarded growth along (121) and the habit change.

For dye-included **UAD** crystals, the absorbance spectra along *a* and *b* are nearly identical for both polarization directions. This indicates that either the dye orientation is nearly 45° or disordered (either statically or dynamically) over a range of angles. Dye inclusion at 45° can achieve hydrogen bonding for both of its amino groups. If the first of the dye's amino groups forms an H-bond with a water molecule at an O···H−N angle of 172° (O···N = 2.8 Å), the second amino group can bond to O8 of the overlying uric acid with an O···H−N angle of 138° (O···N = 3.5 Å). The first corresponds well with the equivalent uric acid−water hydrogen bond, which has an O···H−N angle of 170° (O···N = 2.73 Å).[5] The normal bond distance for the second site is 2.70 Å (O···O) with an O···H−O angle of 145°.

The expanded distance between uric acid layers in **UAD** also enables the acridine to better "fit" within a layer, such that any steric perturbations to the surrounding layers are minimized. Perhaps more importantly, both amino groups of the dye occupy positions where they can form hydrogen bonds with the neighboring molecules and are not presented on the surface in a way that can potentially interfere with the attachment of subsequent material through that crystal face. This is consistent with the fact that no habit changes in dye-included **UAD** matrixes were observed over a broad concentration range.

Conclusions

We have demonstrated that the presence of acriflavine and/or proflavine in solution during uric acid crystal growth results in oriented inclusion of low concentrations of the dye(s) in the crystal matrix. In **UA** crystals, acriflavine is predominantly localized in the {001} and {201} growth sectors and the growth rate of (121) crystal faces is increasingly inhibited by higher solution dye concentrations. In the presence of proflavine, **UA** growth was severely inhibited.

Though acriflavine neutral molecules are oriented in **UAD** matrixes, they do not exhibit a strong preference for a particular growth sector. The orientation of the included dye molecules was determined from linear dichroism measurements and correlated with the host crystal structure. Inhibition of growth along [121] in **UA** is justified on the basis of the proposed inclusion model.

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