Intensities of Calcium Dipicolinate and *Bacillus subtilis* Spore Raman Spectra Excited with 244 nm Light

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Ultraviolet (UV) resonance Raman spectra of *Bacillus subtilis* endospores have been excited at 244 nm. Spectra can be interpreted in terms of contributions from calcium dipicolinate and nucleic acid components. Differences between spectra of spores and vegetative cells are very large and are due to the dominance of the dipicolinate features in the spore spectra. Because the DNA and RNA composition of *B. subtilis* spores is known and because the cross-sections of Raman bands belonging to DNA and RNA bases are known, it is possible to calculate resonance Raman spectral cross-sections for the spore Raman peaks associated with the nucleic acids. The cross-sections of peaks associated with calcium dipicolinate have been measured from aqueous solutions. Cross-section values of the dominant 1017 cm^{-1} calcium dipicolinate peak measured from the Bacillus spores have been shown to be consistent with a calcium dipicolinate composition of ten percent or less by weight in the spores. It is suggested that spectral cross-sections of endospores excited at 244 nm can be estimated to be the sum of the cross-sections of the calcium dipicolinate, DNA, and RNA components of the spore. It appears that the peaks due to DNA and RNA can be used as an internal standard in the calculation of spore Raman peak cross-sections, and potentially the amount of calcium dipicolinate in spores. It is estimated on the basis of known nucleic acid base cross-sections that the most intense Raman band of the *Bacillus subtilis spore* spectra has a cross-section of no more than 4×10^{-18} cm^2/mol-sr.

Index Headings: **Ultraviolet; Raman spectroscopy; Cross-section; Bacillus; Spore.**

INTRODUCTION

Several studies have shown that intense characteristic resonance Raman spectra[1–5] of bacterial vegetative cells can be excited in the region 200–257 nm. Raman spectra[3] excited at 218–231 nm reflect aromatic amino acid contributions, while in spectra excited at 242–257 nucleic acid base modes predominate. Raman spectral cross-sections have been measured[6,7] for 229, 244, 248, and 251 nm excited spectra. It has been shown[6] that nucleic acid Raman cross-sections of *E. coli* vegetative cells can be approximated as the sum of the cross-sections of aromatic amino acid and nucleic acid spectra. Cell spectral intensities have been predicted on the basis of known nucleic acid and amino acid compositions of *E. coli* vegetative cells in the logarithmic growth phase. Modest amounts of hypochromism have been observed for nucleic acid spectra, and strong hyperchromism has been noted in the protein spectra.

It is known[8–10] that Bacillus spores contain significant amounts of calcium dipicolinate (CaDP). Concentrations in spores are so high (5–15%) that dipicolinate peaks have been detected[11] as features of conventional Raman spore spectra excited in the visible range. The dipicolinate spectral features have been shown to be strongly resonance enhanced[12] with 222–251 nm excitation. Dipicolinate spectral features predominate in spore spectra excited by 242 nm light. The Raman and infrared (IR) spectra of dipicolinic acid and calcium dipicolinate have been subject to careful analysis.[13] Consequently, peak assignments can be made with confidence.

Because rapid detection and quantitation of Bacillus spores is an important current public health issue, recently[14–24] a number of approaches to the rapid detection of spores have been pursued. Ultraviolet (UV) resonance Raman detection of spores is especially attractive because of the potential single-spore detection limits[25] and because of the potential for remote detection using laser-optical methods.

Spores belonging to the genus Bacillus have been studied in detail, but the spores of *Bacillus subtilis* are among the most extensively studied. Each spore[28] is known to contain one genome, the mass and composition of which[27] has been established. Average RNA compositions can be predicted[27] if growth conditions have been established. Calcium dipicolinate amounts[9] can be predicted as well, although values can vary depending upon cultural conditions.

An earlier study[12] has shown that 242 nm excitation of Bacillus spores produces spectra containing two very intense peaks. One, which occurs at 1017 cm^{-1}, is due entirely to dipicolinate. A broader peak at 1487 cm^{-1} is due to adenine and guanine components of RNAs and DNA. In this study the Raman spectral cross-sections of all the prominent peaks of the 244 nm excited resonance-enhanced Raman spectra will be calculated. It will be of special interest to determine if the cross-sections of the spore Raman peaks can be calculated based on the measured cross-sections of calcium dipicolinate and the nucleic acid component peaks given the known ranges in amounts of these materials in spores.

EXPERIMENTAL

Chemicals. Dipicolinic acid (DPA) was purchased from Sigma Chemical Company. Calcium dipicolinate solutions were prepared by mixing equal molar amounts amounts of DPA, $CaCl_2$, and NaOH.

Microbiological Samples. For the production of *B. subtilis* endospores bacteria were grown on nutrient agar slants at 30 °C for 7 days. Bacteria were removed and placed in distilled water. Osmotic pressure lysed the vegetative cells. Endospores were pelleted by centrifugation at 12 100 g for 10 min. The pellet was washed in distilled

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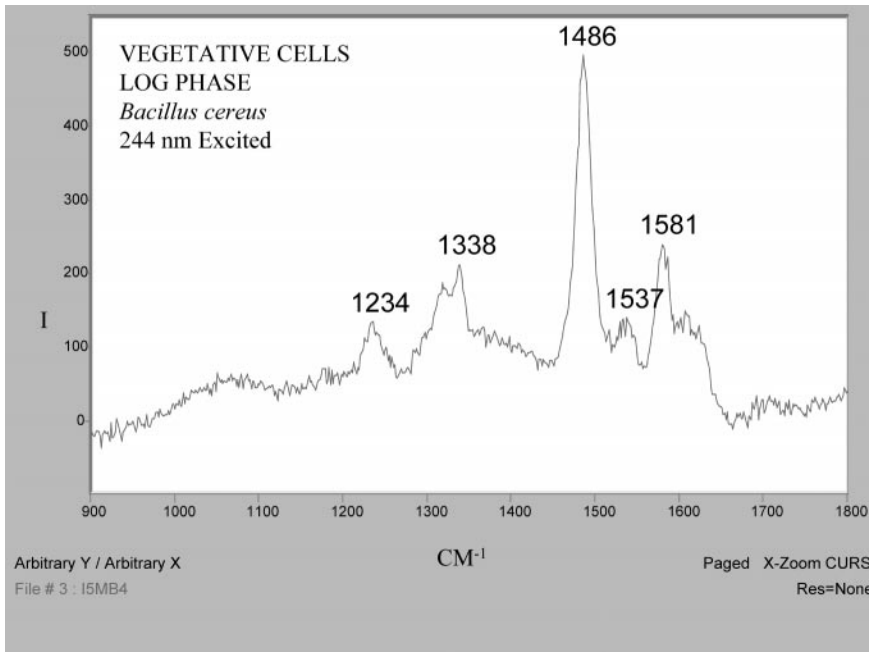

FIG. 1. UV resonance Raman spectrum of *Bacillus cereus* vegetative cells excited by 244 nm light.

water and re-suspended in distilled water. The suspension contained 99% endospores by microscopic observation. The endospores of *B. cereus* were prepared in similar fashion.

Instrumentation and Spectral Measurement. Raman spectra were obtained with a Lexel Model 95 continuous wave (cw) Argon Ion Laser equipped with an extended cavity. Intercavity frequency doubling with a BBO crystal allowed excitation at 244 nm. A flow system circulated the sample at a rate of 60 mL/min by means of a Masterflex pump in a closed loop consisting of the pump and a flat synthetic quartz sample cell with a path length of 5–8 mm. The laser beam impinged on the flat quartz cell at an angle of 30° to the optic axis defined by the spectrometer, to avoid collecting reflected laser light. The power of the light at the cell surface was approximately 5 mW. The Raman signal was collected and depolarized by means of a quartz lens and prism, respectively, located along the direction perpendicular to the sample surface. A 150 μm exit slit was used throughout, which corresponded to a spectral slit of 8 cm^{-1}. Collection times were 60, 120, or 180 s. At least three spectra were taken of each sample. A Spex 1000M, 1 meter, single-grating spectrometer was equipped with a 2400 grids/m grating. This produced a dispersion of 0.4 nm/mm across an 1100B, Princeton Instrument, Inc., charge-coupled device (CCD) camera, which was cooled by liquid nitrogen. A solid edge filter (Barr Associates, Inc., Westfield, MA) rid the spectra of most Rayleigh scattering interference and provided high throughput (90–95%) for Raman lines. The incident laser beam was estimated to give a spot size of 10 × 2 to 50 × 50 μm at the sample cell. The Raman shift axis was calibrated with pure ethanol. All raw data were collected digitally and imported into GRAMS 32 software (Galactic Industries, Inc., Salem, NH) for processing and display. Peak heights, areas, and baselines were determined with the aid of GRAMS 32 software for all spectra. Since spectral bandwidths rarely were dominated by the instrument band-pass, spectral peak areas were used in all intensity calculations. Background subtraction was used to remove contributions from water and the quartz cell. Background spectra were obtained always keeping the cell and optics in a position exactly identical to that of the sample.

Raman Spectral Cross-Section Calculations. Raman cross-sections were determined by direct comparison with that of a sulfate internal standard[28,29] established previously. The cross-section of an analyte peak is taken as proportional to the integrated intensity of the sulfate 981 cm^{-1} peak. The relation between the Raman cross-section of the analyte and the internal standard peaks are defined by the equation:

$$\sigma_N = \sigma_S(I_N/I_S)(C_S/C_N)\{(\nu_o - \nu_s)/(\nu_o - \nu_N)\}^4$$

The Raman cross-section σ_N of a Raman band of the analyte at a frequency ν_N is determined by comparison of its integrated peak intensity, I_N, with the peak intensity, I_S, of the internal standard of known absolute Raman scattering cross-section (σ_S) at frequency ν_S. C_N and C_S are the molar concentrations of the analyte and standard, respectively. The laser frequency is ν_o. The absolute Raman cross-section of the sulfate 981 cm^{-1} symmetrical stretching vibration has been determined previously[28,29] as a function of the UV excitation wavelength. Spectra of the analytes were taken both with and without the presence of the internal standard to separate the contributions of the sulfate peak from those of adjacent analyte peaks.

RESULTS

Figure 1 shows the UV resonance Raman spectrum of *B. cereus* vegetative cells in the logarithmic growth phase. Peaks are due nearly entirely to nucleic acids. The intense peak at 1486 cm^{-1} reflects enhanced rRNA amounts and is due primarily to adenine and guanine. The

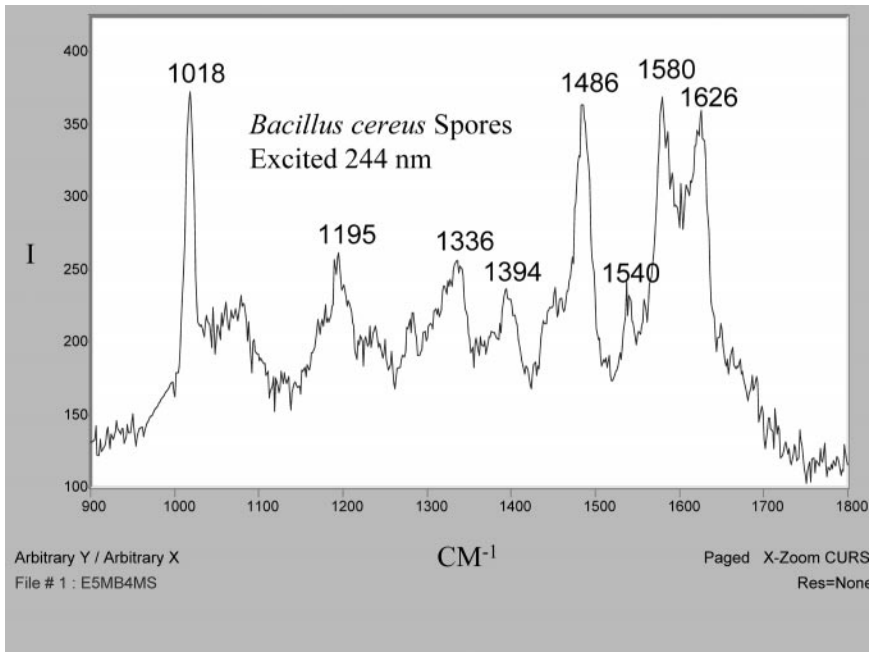

FIG. 2. UV resonance Raman spectrum of *Bacillus cereus* endospores excited by 244 nm light.

peak at 1581 cm^{-1} reflects contributions from guanine and adenine as well. The weaker peak at 1234 cm^{-1} is attributed to a mix of uridine and thymidine, while the 1338 cm^{-1} peak is due primarily to adenine. The small peak at 1537 cm^{-1} is due to cytosine alone. All of the peaks seen in the vegetative cells are noted in the spore spectrum presented in Fig. 2.

The spore spectra are much more complex. The added peaks are due nearly entirely to the presence of calcium dipolinate (CaDP) in the spores. Figure 3 shows the spectrum excited from solutions of calcium dipicolinate. Peaks easily distinguished from nucleic acid peaks occur at 1018, 1280, 1396, and 1445 cm^{-1}. All these peaks are identified easily in the spore spectra. The peak at 1018 cm^{-1} is especially strong and diagnostic for spores. Dipicolinate peak assignments have been discussed previously.[13] Raman spectral cross-section results derived from Fig. 3 are presented in Table I. That the resonance enhancement of the 1018 cm^{-1} peak is about 100-fold can be observed directly from the spectral peak heights, widths, and solute concentrations. From the cross-section for the CaDP and the known amount of CaDP per spore, cross-sections for the spore CaDP peaks can be calculated. It is assumed in the calculations that both hypo- and hyperchromism can be ignored. The calculations follow.

The expected mass[9] of CaDP in a *Bacillus subtilis*

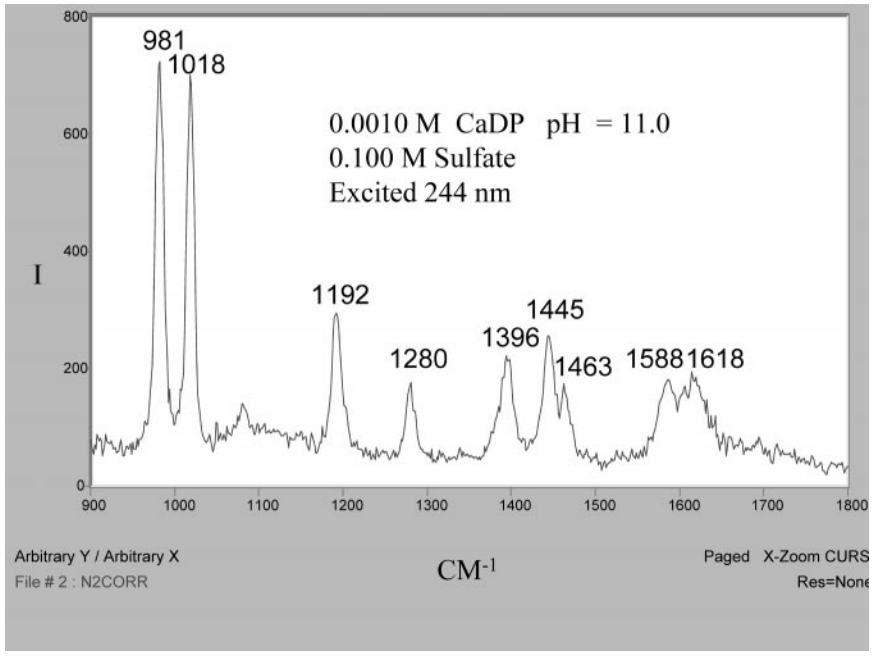

FIG. 3. UV resonance spectrum of 0.0010 M calcium dipicolinate solutions in 0.100 M sodium sulfate excited by 244 nm light.

TABLE I. Experimental cross-sections of prominent calcium dipicolinate Raman bands excited from aqueous solution by 244 nm light.

Raman bands cm^{-1}	1018	1192	1280	1396	1445–63
Cross-sections ($\times 10^{-27}$ cm^2/mol-sr)	21	9.5	4.3	9.5	12.8

TABLE II. Spore estimated cross-sections based on the use of nucleic-acid-base peak cross-sections as internal standard.

Raman bands cm^{-1}	1017	1195	1336	1394	1486
Cross-sections ($\times 10^{-18}$ cm^2/mol-sr)	2.2	2.0	2.5	1.0	4.0
Peak components	CaDP	CaDP	A	CaDP	A+G

spore is about 5×10^{-14} g. Given a cross-section of 0.256×10^{-25} cm^2/mol-sr for a CaDP molecule, and a 10% by mass composition CaDP (5×10^{-15} g), the cross-section for the 1018 cm^{-1} peak is calculated to be about 4×10^{-18} cm^2/mol.-sr.

The cross-section of the nucleic acid peaks can be approximated by adding the contributions of A+G bases in both DNA and RNA. Spore DNA and RNA amounts[27] have been established. The presence of one genome per spore is established[26] for *B. subtilis,* and the ratio[27] of RNA/DNA nucleosides is about 4.5/1. GC/AT molar ratios are known[27] as well for both RNA and DNA components of *Bacillus subtilis.* For example, the DNA contains 1.6×10^6 units of G and 2.4×10^6 units of A. For RNA there are 10×10^6 units of G and 8.2×10^6 units of A, giving a total for the spore of G (11.6×10^6) and A (10.6×10^6) units. At 244 nm the cross-sections of the 1485 cm^{-1} peak has been determined[30] for G and A, respectively, as $\sigma_G = 237 \times 10^{-27}$ cm^2/mol-sr and $\sigma_A = 107 \times 10^{-27}$ cm^2/mol-sr. The molecular spectral cross-section of the 1485 cm^{-1} spore peak calculated as the sum of molecular cross-sections is 4×10^{-18} cm^2/mol-sr. This value is close to the value calculated for the 1018 cm^{-1} peak of CaDP in *B. subtilis* spores based on the 10% mass of CaDP per spore.

The *Bacillus subtilis* spore spectrum (Fig. 4) shows that the 1017 and 1486 cm^{-1} peaks are of nearly identical height and are of similar area. This becomes even more apparent when the contributions of the 1445 and 1463 cm^{-1} peaks of CaDP are subtracted from the broad 1486 cm^{-1} A+G peak. Spore Raman cross-sections based on the nucleic acid 1485 cm^{-1} peak as internal standard appear in Table II.

Previous UV Raman studies[6,7] of bacteria have shown that nucleic acid peaks excited at 244 nm can be approximated as the sum of A and G base molecular spectral contributions. Bacterial Raman cross-sections showed a modest degree[6] of hypochromism. Spectra of *B. subtilis* vegetative cells excited at 244 nm have been published[7] previously.

The observation in the *B. subtilis* spore spectra (Fig. 4, Table II) that the cross-sections of the 1018 cm^{-1} peak of CaDP and the 1485 cm^{-1} peak due to A+G are similar in magnitude is of considerable interest. Nearly equal peak intensities for the 1017 and 1485 cm^{-1} peaks are expected on the basis of the cross-section calculations outlined above. This supports the suggestion that the spore Raman peak intensities based on the spore A+G composition of the spore can be used as an internal standard in the study of spore spectra. The 1018 cm^{-1} peak due to CaDP is somewhat less intense (2.2×10^{-18}) than the expected value (3.2×10^{-18}) based on the original calculations. This difference may be due to the spore containing less than 10% CaDP, or due to CaDP hypochromism. It could also be due to an underestimation of the amount of RNA in the spores or an overestimation of the average mass of the spore itself. Since the bacterial spectra come from the same RNA and DNA material as observed in the spores, it is likely that the nucleic acid cross-sections per G or A unit in spores will not be sig-

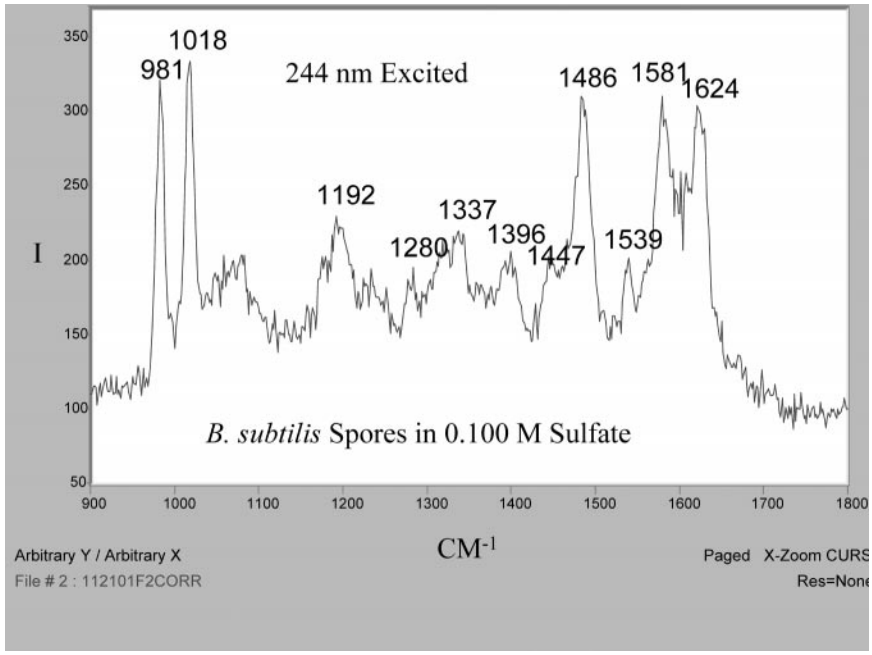

FIG. 4. UV resonance Raman spectrum of *Bacillus subtilis* endospores in 0.100 M sodium sulfate excited by 244 nm light.

nificantly different for those of bacteria. Ratios (891 cm^{-1}, sulfate/1018 cm^{-1}, CaDP) of peak areas show that concentrations of CaDP in the aqueous solution of Fig. 3 and the spore suspension of Fig. 4 are very nearly equal (0.001 M).

In an earlier study of bacteria,[7] the 1485 cm^{-1} peak cross-section for the stationary-phase bacteria (which contain at least two copies of the DNA as opposed to one for the spore, and which are expected to have modestly greater amounts of rRNA) has been shown to have a value of 7.9×10^{-18} cm^2/mol-sr. The observation that the spore has an (A+G) 1485 cm^{-1} cross-section roughly half that of a vegetative cell is a reasonable one. On the basis of these calculations and observations we expect an upper limit to the *Bacillus subtilis* spore 1485 cm^{-1} spectral cross-section to be about 4×10^{-18} cm^2/mol-sr. The intensity of the CaDP 1018 cm^{-1} peak of the spores is directly proportional to the amount of CaDP present. Data suggest that the spores studied have a CaDP composition of 7%.

CONCLUSION

High-quality UV resonance Raman spectra can be excited by 244 nm light from both spores and vegetative cells of *Bacillus cereus.* Spore spectra for *Bacillus cereus* and *Bacillus subtilis* are very similar. Spectral differences between the spores and their corresponding vegetative cells have been shown to be very large. The spectral cross-section of the CaDP 1018 cm^{-1} mode measured from CaDP solutions is enhanced by a factor of about 100 with 244 nm excitation. The cross-section measured for CaDP solutions can be used to calculate an estimated cross-section of the corresponding 1018 cm^{-1} peak belonging to the *B. subtilis* spores given a known CaDP amount per spore. The similar 1018 cm^{-1}/981 cm^{-1} peak intensity ratios observed in Figs. 3 and 4 suggest that the total concentration of CaDP in the spore sample excited is very close to 0.001 M.

Calculations of the 1485 cm^{-1} peak cross-sections for the *B. subtilis* spore have been accomplished by using molar cross-sections for A and G and the known number of A and G units per spore. The calculated values of the CaDP 1018 cm^{-1} peak and the A+G 1485 cm^{-1} peak indicate a CaDP composition of less than 10%. A more likely value is 6–7%. The agreement between calculated and measured cross-sections of spectra of bacterial cells and spores suggests that hypochromism does not effect magnitudes and that estimates of component mass amounts are not in major error. Results suggest that the nucleic acid peaks of spores and perhaps other microbial species can be used as an internal Raman intensity standard in cross-section determinations. Before this is done, however, better values of the hypochromism need to be determined.

Most significantly, it appears that the spore spectral cross-sections can be approximated as the sum of molecular-component cross-sections. These calculated values, made assuming no hypochromism in the nucleic acid spectra, are likely upper limits and not true observed cross-sections, since nucleic acids Raman spectra have been observed[6,31,32] to exhibit various amounts of hypochromism. While the hypochromism observed for the 1485 cm^{-1} mode of *E. coli* is not large,[6] very significant amounts of hypochromism have been observed in virus nucleic acids.[31,32] Another potential source of error involves the way in which the CaDP cross-section is measured. CaDP in the spore is in a very different environment than in aqueous solution. There could be a very significant difference in cross-sections in the two environments.

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