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# Intensities of *E. coli* Nucleic Acid Raman Spectra Excited Selectively from Whole Cells with 251-nm Light

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***Escherichia coli* bacteria in the logarithmic growth phase have been investigated by UV resonance Raman spectroscopy. Bacterial whole-cell Raman spectra excited at 251 nm reflect nearly exclusively the nucleic acid composition even though a very large fraction of the bacterial mass is composed of protein. It has been demonstrated that if bacteria are grown under controlled (logarithmic growth) conditions, which give rise to organisms of known average biochemical composition, the intensities of *E. coli* Raman spectra can be explained quantitatively from the knowledge of component nucleic acid base resonance Raman cross sections.**

Conventional Raman spectroscopy is well suited for studying biological molecules because of its high spectral information content and because water does not interfere significantly. Resonance Raman spectroscopy like conventional Raman is easily applied to aqueous solutions but offers different information. The higher sensitivity and selectivity provided by resonance enhancement may more than offset the loss of spectral information.

Applications of UV Raman to the study of biomolecules have been reviewed[1−8] extensively. Central to the progress in these Raman studies has been the analysis of spectra of purine and pyrimidine bases[9−20] and aromatic amino acids.[5,8,11,14,21−25] Such studies have included the measurement of UV Raman spectral cross sections over a wide range of UV excitation wavelengths. Below 257-nm excitation there is very little fluorescence interference. Resonance enhancement can be very significant. The perils of using pulsed sources of high peak energy have been noted,[3] but most concerns regarding saturation effects and the formation of transients by UV light have been addressed successfully through the use of CW and high duty-cycle pulsed sources of low average power.

This laboratory has pioneered the use of UV resonance Raman spectroscopy in the study of living cells, especially those[26] of bacteria. A substantial part of this work[27] has been reviewed. Spectra have been interpreted primarily on the basis of contributions[28,29] from nucleic acid bases and aromatic amino acids. Changes in nucleic acid contribution with cultural conditions[30] have been observed directly. G−C/A−T molar ratios[30] have been measured spectrally and have been related to the genus to which the bacteria belong. Spectra of dipicolinic acid have been ob-

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served[31] selectively in several types of bacterial spores. Bacterial spectra are sufficiently intense to allow the successful excitation[32] of a very small number of bacterial cells in a Raman UV microscope.

More recent notable success has been obtained elsewhere[25,33,34] in the quantitative UV resonance Raman study of viruses. In the virus studies, significant information regarding the structure, interactions, and dynamics of nucleic acids has been obtained from UV Raman studies. At present, little quantitative UV Raman spectral intensity data have been obtained from bacteria. However, now, given the availability of a CW UV laser, a high duty-cycle picosecond UV laser source, and a sensitive CCD detector, it is possible for us to collect rapidly much more accurate spectral intensity data.

Quantitative Raman intensity data from bacteria are needed if the sensitivity of UVRR spectroscopy is to be determined in bacterial detection. Such data are critical to the successful design of optical instruments proposed for rapid remote bioagent detection. In this work, the bacterium *Escherichia coli* has been investigated by UV resonance Raman spectroscopy with the excitation wavelength of 251 nm. Specifically, an attempt is made to determine whether bacterial spectra can be described quantitatively in terms of the sum of bacterial spectral components. The use of 251-nm light for excitation is ideal for the purpose since protein excitation is minimal at 251 nm. Consequently, bacterial spectra are due nearly entirely to nucleic acids. *E. coli* is a desirable bacterium for study because its cellular composition, and especially its nucleic acid composition, is well known for cultures grown under defined conditions.

If reproducibility in the composition of bacterial cells is desired, organisms in the logarithmic growth stage are convenient to use. Typically, the chemical composition of log-phase *E. coli* dry mass shows[35] 55.0% protein, 20.5% RNA, 3.1% DNA, and 20.6% of other constituents including lipid, lipopolysaccharide, murein, and glycogen, which form the total of 96.1% of macromolecules. The remaining 3.9% include some inorganic ions and various building blocks, metabolites, and vitamins. The average total weight of one cell of *E. coli* in the log phase is $9.5 \times 10^{-13}$ g according to Neidhardt,[35] but the dry weight is $2.8 \times 10^{-13}$ g. The cell contains much water. That nucleic acids can be excited selectively from such a complex mixture is a tribute to the selectivity of resonance Raman detection..

Most significantly the numbers and kinds of nucleic acid base pairs in DNA and RNA are known[35] for log-phase *E. coli*. The Raman spectral intensities for bacteria can be calculated as the sum of spectral intensities of the known numbers and kinds of bases within the nucleic acids. Some uncertainty will be associated with effects such as hypochromism and internal absorption, which will probably vary among nucleic acids within a given cell. This work will attempt to determine whether the UVRR spectral intensities of bacterial *E. coli* can be predicted quantitatively from the knowledge of nucleic acid free base cross sections given the numbers and kinds of base pairs known to be present. If bacteria are grown under controlled conditions, which give rise to organisms of known biochemical composition, this may be possible. Raman spectra of *E. coli* excited at 251 nm have been obtained. The spectral intensities have been measured and compared to the calculated values in this study.

## EXPERIMENTAL SECTION

**Instrumentation** A quasi-continuous third/fourth harmonic tunable, mode-locked coherent Ti:sapphire (Ti:Sa) $Ar^+$ laser system was used as the excitation source, which produces 203-ps pulses at a 76-MHz repetition rate[36] and is continuously tunable over the 200−300-nm range. Raman spectra were taken with excitation at 251 nm. Typical output power was less than 25 mW. A flow system was used to circulate the sample through the laser beam at a flow rate of ∼15 mL/min by means of a Masterflex pump in a closed loop consisting of the pump and a flat quartz sample cell with a path length of about 5−8 mm. The laser beam impinged on the flat quartz cell surface at an angle of ∼30° to the optic axis, defined by the spectrometer, to avoid collecting reflected laser light. The power of the UV light hitting the sample cell surface varied between 8 and 15 mW. The Raman-scattered light was collected and depolarized by means of a quartz lens and prism located along the direction perpendicular to the sample surface. A 150-$\mu$m exit slit was used throughout which corresponded to a spectral slit of 8 $cm^{-1}$. Collection times ranged from 20 to 30 s. Several spectra were obtained from each sample. Typically two 30-s accumulations were added to improve the signal-to-noise ratio. A 1-m-long $f/8$ spectrometer (SPEX 1000M) was used to disperse the light at 0.4 nm/mm with a single 2400 grooves/mm grating across a liquid nitrogen-cooled CCD detector (1100PB, Princeton Instrument, Inc.). A solid edge filter (Barr Associates Inc., Westford, MA) was used to rid spectra of most Rayleigh scattering. At 251 nm, the incident laser beam was estimated to give a spot area of $10 \times 20$ to $50 \times 50$ $\mu m^2$ at the sample cell. The Raman shift axis of the CCD spectra was calibrated by ethanol. All the raw data were collected digitally and imported into GRAMS 386 or GRAMS 32 software (Galactic Industries Co., Salem, NH) for processing and display. Peak heights, areas, and baselines were determined with the aid of the GRAMS 386 or GRAMS 32 software for all spectra. Intensities calculated using peak areas exceeded values calculated from comparison of peak heights typically by ∼15%. Since the spectral bandwiths were not entirely dominated by instrument band-pass, spectral peak areas were used in all nucleoside cross-section and bacterial spectral intensity calculations. Background subtraction was used each time to remove solvent Raman bands as well as spectral contributions due to the quartz cell. Background spectra were obtained always keeping the cell in a position identical to that used to obtain spectra of the nucleosides or bacteria.

**Chemicals** Nucleosides (A, G, U, C, T), aromatic amino acids (Trp, Tyr), $Na_2SO_4$, and $D_2O$ (99.9%) were purchased from Sigma Chemical (St. Louis, MO). Solutions of nucleosides and aromatic amino acids were prepared in the concentration range of 0.2−2.0 mM in $H_2O$.


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**Microbiological Samples.** *E. coli* was obtained from the Microbiology Department, University of Rhode Island, Kingston, RI. Stock cultures were maintained on Trypticase soy agar slants at 4 °C, following incubation at 37 °C for 24 h. A loopful of bacteria was resuspended in 5 mL of nutrient broth and 50 of mL Trypticase soy broth (TSB) without glucose (1.5% Trypticase, 0.5% Soytone, and 0.5% NaCl), and incubated with shaking (250 rpm) for 15−18 h at 37 °C. The bacteria were washed with 4−5 mL of sterile 0.85% saline and then twice centrifuged to remove any culture medium from the bacterial pellet. The bacteria were resuspended in 5 mL of 0.025 M phosphate buffer (pH 6.8) before use.

The bacterial samples were enumerated by three methods. The cell concentration was first estimated by reading the optical density (OD) at 600-nm wavelength with a Baush & Lomb spectrophotometer. A total direct count of a serial dilution was then determined using a standard hemacytometer (Reichert, Buffalo, NY). The number of cells in three squares were counted and then averaged. The concentration was then calculated using the following formula:

$$\text{concentration} = \text{average number of cells/square} \times 16 \times 10^4 \times \text{dilution factor}$$

The third method used was a standard plate count. The number of viable cells was determined by counting the colony-forming units. Serial dilutions were performed to provide between 25 and 250 colony-forming units per milliliter plated. Aliquots were plated and then incubated for 24 h at 37 °C. The concentration of viable cells/ml was then calculated using the following formula:

$$\text{viable cells} = \text{average number of cells} \times \text{dilution factor}$$

The average of the direct count and colony-forming unit count was used in the calculation of the bacterial concentrations used in cross-section determination.

## METHODS OF CALCULATION

**Raman Scattering Cross Section.** The intensity measurements were obtained by direct comparison of analyte Raman peak areas with the Raman spectral area of an aqueous sodium sulfate peak. Use of an internal standard eliminates the need to determine notoriously difficult absolute intensity measurements. In this work, aqueous $Na_2SO_4$ is used as the internal standard in all calculations of Raman spectral cross sections of nucleoside peaks or the relative intensities of bacterial spectral peaks assigned to nucleic acids or aromatic amino acids.

The Raman cross section ($\sigma_N$) of a Raman band of a sample at frequency $\nu_N$ is determined by comparison of its peak intensity ($I_N$) with the peak intensity ($I_S$) of the internal standard of known absolute Raman scattering cross section ($\sigma_s$) at frequency $\nu_S$ by using the equation below, and a method described previously.[22]

$$\sigma_N = \sigma_s(I_N/I_S)\,(C_S/C_N)\{(\nu_0 - \nu_S)/(\nu_0 - \nu_N)\}^4 \qquad (1)$$

$\nu_0$ is the laser frequency and $C_N$ and $C_S$ are the molar concentrations of the sample and standard, respectively.

Table 1. Composition of E. Coli B/r ($\mu$mol/g of Dried Cells)

| | RNA nucleotides | DNA nucleotides | protein amino acid |
|---|---|---|---|
| AMP | 165 | 24.6 | |
| GMP | 203 | 25.4 | |
| CMP | 126 | 25.4 | |
| UMP | 136 | | |
| TMP | | 24.6 | |
| Trp | | | 54 |
| Tyr | | | 131 |

Table 2. Number of Molecules of A, G, C, U, T, Trp, and Tyr Units in One Cell of *E. coli*

| component | molecules $\times 10^6$/cell | component | molecules $\times 10^6$/cell |
|---|---|---|---|
| A | 32.0 | T | 4.15 |
| G | 38.6 | Trp | 9.11 |
| C | 25.6 | Tyr | 22.1 |
| U | 22.9 | | |

The prominent Raman band of the internal standard sodium sulfate at 981 $cm^{-1}$ is assigned to the symmetrical stretching vibration of the $SO_4^{2-}$ ion. The absolute Raman cross section of this band has been determined previously[22,37] as a function of the UV excitation wavelength.

**Calculated Raman Intensities of Nucleic Acid Peaks of Bacteria.** The Raman intensities of the nucleic acid peaks belonging to the bacterial cell have been calculated. This has been done through summation of the cross sections of the known numbers and kinds of bases belonging to the nucleic acids. The number and the composition of bases belonging to DNA and RNA for *E. coli* B/r protoplasm is known[35] on the average for cells in cultures of logarithmic growth phase and is listed in Table 1.

With the information in Table 1 and the knowledge of the average weight of one dry cell of *E.coli* ($2.8 \times 10^{-13}$ g/cell), the number of bases, A, G, C, U, T, and the number of aromatic amino acid units of Trp and Tyr in one *E. coli* cell have been calculated and listed in Table 2.

The Raman spectral intensity of nucleic acid peaks belonging to a single bacterial cell ($\sigma_{cal}$) can be calculated from the summation of the absolute cross sections of the Raman band components ($\sigma_C$):

$$\sigma_{cal} = \sum f_C \sigma_C \qquad (3)$$

where $f_C$ is the number of each component in one cell of *E. coli*. The Raman cross sections belonging to selected modes of adenosine, guanosine, cytidine, uridine, thymidine, and the 1610−1620-$cm^{-1}$ bands of tryptophan and tyrosine have been measured with 251-nm excitation in this work since these are not available in the literature.

The reported Raman cross sections of nucleosides are averages of multiple independent experiments exhibiting band intensity deviations of less than ±10%. The scattering background due to the quartz cell and the solvent was subtracted from all bacterial spectra.

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Table 3. Cross Sections of Prominent Raman Bands of Nucleosides in UVRR Spectra Excited at 251, 252,[15] and 257[25] nm

| Raman band ($cm^{-1}$) | cross section (mbarns) 251 nm | 252 nm | 257 nm |
|---|---|---|---|
| | Adenosine | | |
| 1336 | 439 | 401 | 367 |
| 1482 | 265 | 265 | 211 |
| 1506 | 109 | | 109 |
| 1580 | 98 | 114 | 76 |
| | Guanosine | | |
| 1332 | 187 | | 80 |
| 1361 | 84 | 63 | 36 |
| 1485 | 477 | 391 | 208 |
| 1575 | 227 | 195 | 114 |
| | Cytidine | | |
| 1252 | 20 | | 24 |
| 1293 | 17 | 20 | 20 |
| 1527 | 56 | 47 | 29 |
| 1650 | 45 | 29 | 18 |
| | Uridine | | |
| 1230 | 156 | 135 | |
| 1394 | 37 | 39 | |
| 1476 | 20 | 22 | |
| 1625 | 71 | 51 | |
| | Thymidine | | |
| 1190 | 31 | | 24 |
| 1244 | 70 | | 58 |
| 1374 | 92 | | 85 |
| 1414 | 15 | | 15 |
| 1652 | 117 | | 88 |

Bacterial spectral intensities calculated as the sum of molecular cross sections of nucleosides and aromatic amino acids are expected to differ from values obtained experimentally. Changes in the local molecular environment of bases due to stacking interactions in nucleic acids, for example, frequently lead to a reduction in Raman intensity. This reduction is part of a phenomenon referred to as hypochromism.[38] In contrast, aromatic amino acids in proteins can exhibit significant enhancements of Raman intensity associated with hyperchromism. An excellent discussion[33] of these phenomena observed in viral proteins and nucleic acids has been published.

## RESULTS

Aqueous nucleoside solutions have been excited at 251 nm. The Raman cross sections of modes of adenosine, guanosine, cytidine, uridine, and thymidine have been determined independently and are compared with 252- and 257-nm excitation results determined elsewhere listed in Table 3.

Bacterial Raman spectra excited at 257 and 251 nm are similar in appearance.[27] However, 251-nm excitation provides excellent selectivity in nucleic acid detection and better quality spectra. With 257-nm excitation, aromatic amino acid fluorescence begins to interfere[27] significantly beyond 1000 $cm^{-1}$. In contrast, with 251-nm excitation, fluorescence interference within the fingerprint region is negligibly small.

Raman spectra excited at 251 nm for a series of diluted *E. coli* log-phase suspensions in $H_2O$ and $D_2O$ with sodium sulfate as internal standard are shown in Figure 1 and Figure 2, respectively, with the background subtracted. The measured Raman cross sections for the major peaks of a typical serial dilution experiment involving *E. coli* in aqueous suspension are listed in Table 4.

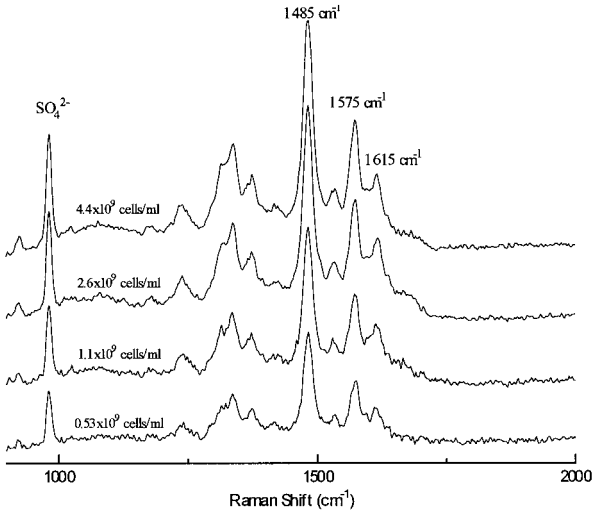


Figure 1. Raman spectra of serial diluted *E. coli* log-phase suspensions in $H_2O$. Sulfate concentrations adjusted for convenience as in Table 4.

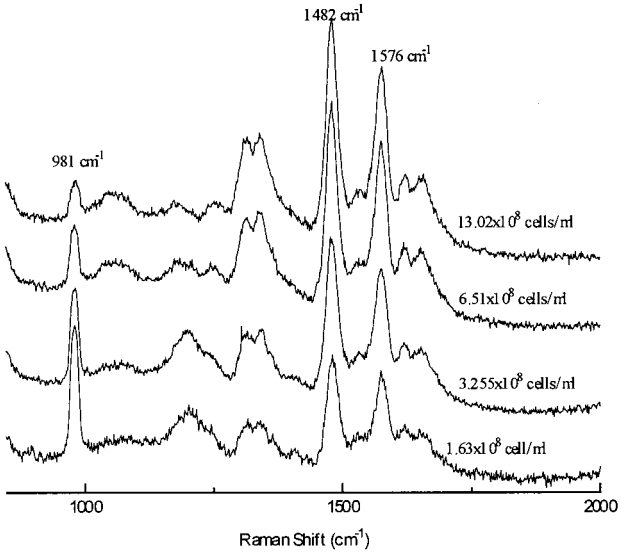


Figure 2. Raman spectra of serial diluted *E.coli* log-phase suspensions in $D_2O$. Sulfate concentrations constant.

Concentrations of sulfate ion typically were varied to give sulfate peaks slightly less intense than the most intense nucleic acid peak for a particular bacterial suspension. In Figure 1, which represents a typical experimental sequence, the intensity of the sulfate peak relative to the nucleic acid peak remains nearly constant. The reduction in the signal-to-noise ratio for the spectra of the more dilute suspensions is evident. In contrast, Figure 2 better represents the change in peak intensities with bacterial concentration. The spectra of Figure 2 reflect constant sulfate concentrations while bacterial concentrations vary significantly.

In Table 5 the average values of the Raman spectral intensities for each major bacterial mode are compared with values calculated from the sum of identical nucleic acid base contributions. No effects due to hypochromism or internal absorption are included in the calculations.

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Table 4. Experimental Raman Relative Intensities of a Typical Serial Dilution of Log-Phase *E. coli* Excited at 251 nm ($6.25 \times 10^8$ Cells/OD)

| | | | intensities (x $10^{-18}$) | | | | |
|---|---|---|---|---|---|---|---|
| OD | conc (M) $\times 10^{-12}$ | $Na_2SO_4$ (M) | 1240 $cm^{-1}$ | 1334 $cm^{-1}$ | 1485 $cm^{-1}$ | 1575 $cm^{-1}$ | 1618 $cm^{-1}$ |
| 0.98 | 1.01 | 0.075 | | 9.61 | 20.0 | 8.72 | 3.68 |
| 1.96 | 2.03 | 0.15 | 3.06 | 8.82 | 22.2 | 8.06 | 4.22 |
| 3.92 | 4.07 | 0.30 | 3.20 | 9.16 | 21.2 | 8.67 | 3.38 |
| 7.84 | 8.14 | 0.40 | 4.01 | 9.10 | 21.0 | 8.66 | 3.10 |

Table 5. Experimental and Calculated Intensities of Prominent Raman Bands for *E. coli* (Log Phase) Excited at 251 nm ($cm^2$/mol·sr $\times 10^{-18}$)

| | Raman bands ($cm^{-1}$) | | | | |
|---|---|---|---|---|---|
| | 1240 | 1334 | 1485 | 1575 | 1618 |
| intensities (measd) | 3.47 | 10.3 | 22.2 | 9.15 | 3.78 |
| peak components | T + U | A | A + G | A + G | Trp + Tyr |
| intensities (calcd) | 3.96 | 13.9 | 27.1 | 11. | 1.3 |

The 1618-$cm^{-1}$ Raman band of the bacteria is composed of tyrosine and tryptophan and a negligible component due to phenylalanine. The cross sections of aqueous tyrosine and tryptophan modes have been measured in this work at 251-nm excitation. The values used to calculate the intensity of this 1618-$cm^{-1}$ peak are 38.5 (Tyr) and 43.7 (Trp) mbarns, respectively.

## DISCUSSION

**Nucleoside Raman Cross Sections.** The data of Table 3 have been obtained since they were crucial to the calculation of the bacterial Raman intensities. Until now there were no such data available with 251-nm excitation. There is substantial advantage for obtaining these data with 251-nm excitation rather than 244- or 257-nm excitation, as is commonly the case due to the strength of the latter laser lines. Given 244-nm excitation of bacterial cells, interference from aromatic amino acid spectra can be quite strong. On the other hand with 257-nm excitation fluorescence, interference begins to come into play, and intensities of at least the 1485-$cm^{-1}$ guanosine line are significantly lower. It was judged risky to use extrapolations of previous data. Hence, the nucleoside spectra were determined directly with 251-nm excitation. In addition, it was believed that values excited at 251 nm should fall close to those excited at 252 nm if we were to be confident in our method of calculating cross sections. Basically, all the lines of high intensity generated by 251-nm excitation lie close to the cross-section values observed previously[15] at 252 nm. In fact, this shows that we could have used the 252-nm excited cross-section data in this work without a change in our conclusions. Our initial fears were unfounded.

**Raman Bacterial Spectral Intensities.** The Raman intensities for the bacterial peaks have been calculated by assuming that the bacterial peaks excited by 251-nm laser light are due to excitations of purine and pyrimidine base contributions from the nucleic acids. For the purposes of comparison with experimental results, the calculated intensities are based upon the assumption that hypochromism and internal absorption can be ignored. Basically, given the known average A, G, C, T, and U content per *E. coli* cell, the intensities have been calculated as the sum of the individual base contributions. It is assumed as well that the corresponding nucleic acid peak positions are very close to one another. That is, it has been assumed that the guanine bases in DNA, for example, will exhibit the same spectral energies as the guanine in RNA. This will probably not be true exactly, but we have assumed this true for the purposes of our calculations. It is expected that, because of the heterogeneity of the cell, spectral lines due to bacteria will show broadening of peaks even beyond that expected due to the resonance nature of the spectra. The bacterial peaks are very close in energy to those of the nucleosides. In the context of this work, it is proposed that bacteria be considered as resembling a giant macromolecule having functional groups of the same kind in different environments. The assumption is a large one, but it seems to hold fairly well here for the purposes of spectral interpretation.

Table 4 shows the measured intensities from one typical *E. coli* log-phase serial dilution experiment. The standard deviation is only 0.88 ($(21.2 \pm 0.9) \times 10^{-18}$ $cm^2$/mol·sr) for the 1485-$cm^{-1}$ Raman band based on the peak intensity data for this typical serial dilution experiment. But for all the multiple independent experiments, the standard deviation is much larger ($(21.2 \pm 5.8) \times 10^{-18}$ $cm^2$/mol·sr) for the same band. Constituents of *E. coli* in the log phase A, G, C, T, and U are well-defined, but in practice, the enumeration of the organisms proved the greatest source of variability. Since only live organisms were counted in the colony-forming unit count, but live and dead organisms will give similar Raman spectra, our cross sections may be too large to the extent that we "undercounted" the cells. It is believed that under the conditions applied very few cells died. There is also the possibility of "undercounting" due to the clumping of bacteria. However, this problem is minimized for *E. coli* since these organisms do not tend to aggregate.

The comparison of the calculated with the experimental intensities in Table 5 shows that experimental values are close to the calculated ones. In addition, the experimental values are modestly lower in magnitude. This lower magnitude of the measured intensities compared to the calculated ones is attributed to hypochromism. Because of the presence of different kinds of nucleic acids contributing to the same spectral intensity, there is no way of calculating the hypochromism due to any one component. Still, so long as there is stacking present in the nucleic acids hypochromism is expected. The amount of hypochromism falls within a range observed previously[25,38] for nucleic acid Raman spectra.

Most significantly, the experimental and calculated intensities are very similar in magnitude. This shows that to a good approximation the intensities of nucleic acid resonance Raman peaks of *E. coli* bacteria excited at 251 nm can be estimated from

cellular nucleic acid base composition. This strongly suggests that the intensities of Raman spectra of other bacterial species can be predicted successfully as well, if the nucleic acid composition is known. Such calculations now allow the direct comparison of the intensities of resonance Raman detection with bacterial detection using other spectroscopic methods.

Spectra of Figure 2 show that modest, but significant spectral changes occur when the bacteria are suspended in $D_2O$. The spectral shifts are similar in magnitude to those reported[34] for viral nucleic acids.

## CONCLUSIONS

High-quality resonance Raman spectra of *E. coli* have been excited by 251-nm light. Spectra show a high selectivity for nucleic acids and the absence of fluorescence in the fingerprint region. High signal-to-noise ratios permit precise quantitative determination of the Raman peak intensities for prominent Raman bands in bacteria due to nucleic acids. The comparison of the measured and calculated values of the intensities of *E. coli* (log-phase) modes shows the experimental values to be ~25% less than the calculated ones. Raman cross sections of nucleoside spectra excited at 251 nm are close in value to that reported[15] at 252 nm. This agreement between our data of standard nucleosides excited at 251 nm with data excited at 252 nm demonstrates that our method of spectral measurement closely approximates the results determined for nucleosides independently in other laboratories.

It has been demonstrated that *E. coli* Raman spectra excited at 251 nm can be approximated as due to the simple sum of purine and pyramidine base Raman contributions. Hypochromism appears to be in the range of 20−30%. Bacterial Raman spectra excited at 251 nm reflect nearly exclusively nucleic acid composition even though a large fraction of bacterial mass is composed of protein. The single protein peak at 1618 $cm^{-1}$ exhibits strong hyperchromism.

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