pH-Dependent Flavin Adenine Dinucleotide and Nicotinamide Adenine Dinucleotide Ultraviolet Resonance Raman (UVRR) Spectra at Intracellular Concentration

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Abstract
The ultraviolet resonance Raman spectra of the adenine-containing enzymatic redox cofactors nicotinamide adenine dinucleotide and flavin adenine dinucleotide in aqueous solution of physiological concentration are compared with the aim of distinguishing between them and their building block adenine in potential co-occurrence in biological materials. At an excitation wavelength of 266 nm, the spectra are dominated by the strong resonant contribution from adenine; nevertheless, bands assigned to vibrational modes of the nicotinamide and the flavin unit are found to appear at similar signal strength. Comparison of spectra measured at pH 7 with data obtained pH 10 and pH 3 shows characteristic changes when pH is increased or lowered, mainly due to deprotonation of the flavin and nicotinamide moieties, and protonation of the adenine, respectively.

Keywords
Ultraviolet resonance Raman, UVRR, flavin adenine dinucleotide, FAD, nicotinamide adenine dinucleotide, NADH, adenine, pH dependence, protonation

Introduction
The molecular characterization of cellular metabolism at sub-microscopic/microscopic resolution and over time has remained a major aim in molecular biology and biochemistry. Important metabolic reactions involve the ubiquitous biomolecules nicotinamide adenine dinucleotide NAD+/NADH as key redox pair, e.g., as co-enzyme in the respiratory chain or in metabolically important signaling processes, and flavin adenine dinucleotide (FAD), which provides the flavin group to many flavoproteins and can exist in different oxidation states. Their many different functions as co-enzymes in a multitude of biochemical pathways lead to the omnipresence of NADH and FAD throughout a cell or tissue. Nevertheless, local concentration, typically in the sub-millimolar range down to tens of micromolar, can vary greatly, depending on organelles and metabolic status, with fluctuating concentration, especially in mitochondria.

Resonance with a particular electronic transition implies extreme selectivity in the Raman experiment, as the resonance Raman (RR) signal of a particular chromophore is many orders of magnitude stronger than the non-resonant spectrum and precludes observation of all other molecular species in the sample. Resonant excitation in the ultraviolet (UV) has been shown to be very useful in the Raman spectroscopic characterization of microorganisms and eukaryotic cells, and UVRR in microscopes for Raman-based imaging of cells and tissues has been shown to be challenging, but feasible. As examples, nucleotides in cells can be visualized, and quinine...
species can be studied in situ. Based on the rich body of knowledge on the resonant Raman spectra of nucleobases and of the molecules NADH and FAD, which contain adenine as nucleobase, there is great potential for their use in UVRR microscopy of cells. Across an animal cell, pH values can vary greatly, depending on the different cellular organelles. As an example, mitochondrial pH is basic and can change with metabolic state and organelle functioning. The pH dependence of the Raman and UVRR spectra of the nucleobases and for NADH has been reported, suggesting the possibility to study and characterize molecular structure and maybe even pH-dependent changes in situ in cellular environments.

Here, we discuss UVRR spectra of FAD, NADH, and adenine obtained at micromolar concentrations that match those in a cell, with the aim of distinguishing between them at different pH values, including higher pH encountered in mitochondria. As all molecules carry adenine as main chromophore that is determining the UVRR spectra at this excitation wavelength (Fig. 1), we try to focus the discussion on an influence on the adenine spectrum by the presence of the flavin and nicotinamide unit, and on their direct contributions to the spectra. The results will help to understand the spectral signatures of adenine-containing molecules in complex environments as they may be encountered in UVRR microscopy.

Figure 1. The chemical structures of (a) FAD, (b) NADH, and (c) adenine at neutral pH with numbering of the atoms for assignment.

Materials and Methods

Adenine (99%), flavin adenine dinucleotide disodium salt hydrate (≥95%) and β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (≥97%) were purchased from Sigma-Aldrich. Aqueous solutions of a concentration of 10^{-4} M were prepared by dissolving the compounds in ultrapure (MilliQ) water and diluted as indicated. A citrate buffer and a sodium carbonate–sodium bicarbonate buffer were used for adjusting the pH to values of 3 and 10, respectively.

Raman spectra were recorded in backscattering geometry in a quartz cuvette with a path length of 1 cm and a stirring magnet. The sample was excited using a diode-pumped continuous-wave solid state laser (Crystal Laser Systems GmbH, Germany) operating at 266 nm with an average intensity on the sample of 2 \times 10^2 W cm^{-2}. Spectra were obtained at the same focus position. Verification that no sample degradation occurred under these conditions was obtained in test measurements over time ranges of 10 min of repeated spectral acquisitions. The Raman scattered light was detected using a Raman spectrometer T64000 (Germany), operated in single-stage mode using an edge filter, with a spectral collection time of 15 s per spectrum. Use of a 2400 L/mm holographic grating yields a spectral resolution of 3.4 cm^{-1} considering the full spectral range. Averages of three spectra were calculated for analysis. Spectra of the cuvette containing water were used for correction of the sample spectra, which
Results and Discussion

Distinction of NADH and FAD at Neutral pH

Figure 2 shows the UV resonance Raman spectra excited at 266 nm of FAD, NADH, and adenine at neutral pH. The concentration of the molecules in the experiments was chosen to be in the biologically relevant range, which lies between $10^{-4}$ M and $10^{-5}$ M for NADH and FAD, respectively. A high similarity of the spectra is found at pH 7, particularly in the region containing the bands at about 1250 cm$^{-1}$, 1310 cm$^{-1}$, 1330 cm$^{-1}$, 1422 cm$^{-1}$, 1480 cm$^{-1}$, and 1505 cm$^{-1}$ that can all be attributed to vibrations in the adenine moiety (Table I), as discussed in previous reports. Although the UVRR spectrum of FAD excited at 266 nm is known to be dominated by the contributions from the adenine part of the molecule, the electronic interaction with flavin (see Fig. 1a for molecular structure) was discussed to be responsible for the characteristic spectrum as well, as clearly seen here when comparing its spectrum with that of pure adenine (Fig. 2, top and bottom). At neutral pH, both FAD and NADH exist in folded forms where the adenine moiety interacts via $\pi$-$\pi$ stacking with the flavin and nicotinamide moiety, respectively. Copeland and Spiro reported a change in relative intensities on FAD compared to the sum of the intensities of adenosine monophosphate and flavin mononucleotide. A main contribution to the changed signal strength can probably be related to the decreased absorption coefficient of adenine in FDA and NADH compared to unbound adenine that we observed in UV–visible absorption spectra (not shown). Although the concentration chosen for the experiment here matches that of FAD in its real cellular environment, the fact that the molecule may be embedded in a protein environment, where stacking effects could be eliminated or replaced by other electronic interactions, may lead to further modification of the excitation profiles of important adenine modes that will change intensity ratios. Moreover, we expect changes in polarity of the environment, both in proteins and membrane regions, e.g., of the mitochondria to affect the RR signals. While only very small effects of stacking on the non-resonant Raman spectrum of NADH in a real enzyme were reported, the effect on the adenine UVRR signals that dominate the spectrum at 266 nm could be significant as well, as suggested by the inability to reproduce the UVRR NADH spectrum in solution by superposition of a spectrum of adenosine monophosphate and methyl nicotinamide at an excitation of 273 nm.

The sensitivity of the spectrum toward the interactions of the adenine unit in the different molecules may on the one hand reveal different environment of each of the chromophores but may as well enable distinction between NADH and FAD. A conspicuous difference between the spectra is the ratio of the relative intensities of the strong adenine CH deformation/C–N mode at about 1480 cm$^{-1}$ and the deformation mode at 1505 cm$^{-1}$. For FAD they have the same intensity, and for NADH they show a signal ratio of approximately two (Fig. 2). In pure adenine (Fig. 2, bottom), a ratio of 1.5 is found, with the frequency of the deformation vibration shifting slightly to lower energy to $\sim$1500 cm$^{-1}$. In addition to the influence of different stacking effects with the flavin and nicotinamide moiety on signal strength, their contributions to the signal $\sim$1505 cm$^{-1}$ in FAD and NADH in addition to the adenine may contribute to the differences here as well. These are, albeit weak at this wavelength and compared to the strong RR from adenine, signals from the flavin ring I (Fig. 1a) in FAD and from deformation of the ribose unit $R_1$ of the adenine nucleotide in NADH (Fig. 1b).

Different from pure adenine at this neutral pH value, FAD and NADH show a strong, sharp signal at 1579 cm$^{-1}$ (Fig. 2). This band can be assigned to a combination of an adenine vibration and a deformation vibration of the ribose directly connected to the adenine moiety in NADH and other related compounds.

Figure 2. UV resonance Raman spectra of $10^{-4}$ M FAD, NADH, and adenine as indicated at neutral pH ($\lambda = 266$ nm, $3 \times 15$ s). Scale bars: FAD and NADH 500 counts, adenine 2000 counts.
Table I. Tentative assignments for the bands of adenine, NADH, and FAD observed at the different pH values, based on the references cited below.

| Raman shift (cm⁻¹) | Band assignments | NADH | FAD |
|-------------------|------------------|------|-----|
| 609               | Adenine def R6/R5 |      | Adenine def R6/R5 | R6/R5 |
| 620               | Def, in-plane in R6 and R5⁴¹ |      | flavin δ(CN3C), δ(NC5aC6)⁴⁰ |
| 638               | Adenine/R₁³⁴     |      | Adenine/R₁³⁴     |
| 720               | −δN7C8N9-N9R + δC5N7C8 + C4N9C8¹⁸ |      | Adenine |
| 730               | Adenine          |      | Adenine          |
| 790               | Adenine          |      | Adenine          |
| 945               | Def R5 (sqz group N7–C8–N9)⁴² |      | v(C7 methyl), δ(C4=O)²⁸ |
| 1015              | In-plane NH₂ rock⁴² | Adenine | Adenine |
| 1064              | diphosphate³⁴     |      | Adenine |
| 1070              | Diphosphate      |      | Diphosphate      |
| 1223              | nicotinamide NH₂ rock²⁵ |      | Adenine |
| 1227              | ν(C5–N7),⁴¹      |      | Adenine |
| 1249              | ν(C5–N7), bend(C5–N7), N9–H⁴¹ |      | Adenine |
| 1252              | bend C8-H, N9–H, str N7–C8⁴² |      | ν(C6C7), ν(C8–methyl)²⁸ |
| 1305              | Nicotinamide + adenine³⁴ |      | v(C6C7), v(C8–methyl)²⁸ |
| 1310              | ν(C5C7 + N1C2 – C4C5 + δC8H²⁰ |      | Adenine |
|                   | str C2–N3, N1–C2, C5–C6, C5–N7⁴² |      | Adenine |

(continued)
### Table I. Continued.

| Raman shift (cm⁻¹) | Band assignments | NADH | FAD |
|-------------------|------------------|------|-----|
| ~1330             | Bend C2–H, C8–H, N9–H, ν C6–N1, C8–N9, N3–C4, def R5, R6, bend C2–H, C8–H, N9–H, N10–H11 | Adenine/įR₁<sup>34</sup> | Adenine<sup>43</sup> |
| 1352              | bend C2–H, N9–H, ν(C8–N9), C4–N9<sup>42</sup> | Adenine/R₁ + nicotinamide<sup>34</sup> | Ring asymmetric įCH of nicotinamide<sup>25</sup> |
| 1364              | C4N9 + N7C8 + őN9H = ᵃC2H<sup>20</sup> | Adenine/R₁ + nicotinamide<sup>34</sup> | Adenine/flavin ν(C5a–C6) ν(N10C10a)<sup>28</sup> |
| 1422              | C4N9 – őC8H<sup>18</sup> C4C5 – C4N9 – őC2H + őN9H<sup>20</sup> | Adenine/įR₁<sup>34</sup> | Adenine<sup>56</sup> |
| 1480              | –őC2H–N9C8 + őCBH<sup>18</sup> | Adenine<sup>34</sup> | Adenine<sup>25</sup> |
| 1505              | In-plane deformation<sup>24</sup> ν(C6–N10), ν(C2–N3), ν(C6–N1), bend C2–H<sup>42</sup> | Adenine/įR₁<sup>34</sup> | Adenine<sup>43</sup> |
| 1550              | def R5, R6<sup>41</sup> | Adenine (C5C4, C4N3<sup>18</sup>) nicotinamide, ring ν(C=–C) out-of-phase phase<sup>25</sup> | Adenine (C5C4, C4N3<sup>18</sup>) |
| 1558              | NH₂ bend, N1H<sup>34</sup> | Adenine/įR₁<sup>34</sup> | Adenine (ν(N3–C4), ν(N1–C6), ν(C5–N7)<sup>42</sup> fluor ring<sup>19</sup> N(C8C9), ν(C9C9a)<sup>28</sup> |
| 1577              | sciss NH₂<sup>42</sup> | Flavin ring<sup>29</sup> adenine band<sup>46</sup> | |
| 1600              | Adenine (ν(N3–C4), ν(N1–C6), ν(C5–N7)<sup>42</sup> nicotinamide, ring ν(C=–C) in-phase<sup>25</sup> | |

Bend: bending; breath: breathing; def: deformation; rock: rocking; wag: wagging; sciss: scissoring; ν: stretching; R5: five-membered ring; R6: six-membered ring.

Note: Numbering of atoms and molecule subunits referred to is shown in Fig. 1.
in the case of the FAD to a ring vibration of the flavin moiety\textsuperscript{29} (Table I). It has also been observed in other adenine derivatives that is modified at the N9 atom, specifically in adenosine and 9-methyladenine.\textsuperscript{18,21,47} as well as in adenine at acidic pH, where the molecule is fully protonated\textsuperscript{24} (see also discussion below). The spectra reveal further differences that are related to the fact that vibrations involving the N9 atom of adenine are affected by the ribose unit that is connected to this atom in FAD and NADH (Figs. 1a and 1b). An example is the shift of the band assigned to the asymmetric CH deformation mode of the ring at 1364 cm\textsuperscript{-1} in the spectrum of adenine to 1373 cm\textsuperscript{-1} in the spectra of NADH and FAD, respectively. Similar differences were reported for the comparison of the spectrum of adenine and 9-methyladenine.\textsuperscript{47}

Also, vibrations of lower frequencies appear to show characteristic intensity and position depending on the adenine-containing molecular species. The ring breathing mode, observed at 720 cm\textsuperscript{-1} in pure adenine has higher frequency in NADH and FAD (Fig. 2). Although assignment of a small signal at 790 cm\textsuperscript{-1} observed only in the spectrum of FAD may not be fully unequivocal due to incomplete correction of a signal of the material of the optical cell,\textsuperscript{35} its presence here suggests superposition of an out-of-plane mode of adenine by a band assigned to the C7 methyl stretch and the C4=O deformation in the flavin (Fig. 1a).\textsuperscript{28,40} Deformation vibrations of the C=O groups of ring III\textsuperscript{28} of the flavin molecule (Fig. 1a) also cause another signal in FAD at 685 cm\textsuperscript{-1} that is unique among the three molecules (Fig. 2). The strong band at 609 cm\textsuperscript{-1} in the spectrum of FAD is assigned to deformation vibrations in ring I and III of flavin\textsuperscript{28} and can therefore also be considered unique of FAD. Further characteristic assigned to the flavin residue is the band at 1160 cm\textsuperscript{-1} of C6-H deformation and C7 methyl stretching of flavin ring III\textsuperscript{28} that is clearly separated from the in-plane vibration of adenine at about 1177 cm\textsuperscript{-1} common to FAD and NADH (Fig. 2, top and middle). In NADH, a vibration at 632 cm\textsuperscript{-1}, assigned to adenine coupled to the ribose R\textsuperscript{1}\textsuperscript{34} (Fig. 1b) is distinctive as well. The signals characteristic of FAD and NADH can also be identified in average spectra calculated from the FAD and NADH spectra (cf. Fig. S1 for an example), where the absolute signals obtained from a solution of NADH are approximately half of those obtained for FAD at the same concentration (compare scale bars in Fig. 2), also in agreement with the lower molar absorption of adenine in NADH compared to FAD.

Effects of High and Low pH

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Although a comparison of the spectra at neutral pH may be a good approximation of typical environments in tissues and cells, the basic pH of the mitochondrial matrix and the low pH in highly acidified endolysosomes or interstitial fluids that may be encountered as well necessitate discussion of the spectra at basic and acidic condition. Figure 3 compares the UVRR spectra of adenine, NADH and FAD at neutral pH (Figs. 3a to 3c, middle traces) with those obtained in acidic (pH3) and basic (pH 10) solution, chosen here as extreme examples to underpin the effects of protonation and deprotonation of the molecules. At high pH (Figs. 3a to 3b, top traces), a number of well-characterized changes are found in the spectrum of adenine (Fig. 3a), specifically, several bands intensify, e.g., at 1129 cm\textsuperscript{-1} or arise, e.g., at 1196 cm\textsuperscript{-1}, caused by a downshift of the band at 1227 cm\textsuperscript{-1}\textsuperscript{28} or also at 1448 cm\textsuperscript{-1} and 1539 cm\textsuperscript{-1}. It is known that adenine is deprotonated at the N9 position with a pK\textsubscript{a} of 9.8\textsuperscript{46} which implies that at pH 10 nearly all adenine molecules should be deprotonated at N9 (Fig. 1c).

The signal ratio of the two bands at 1329 cm\textsuperscript{-1} and 1310 cm\textsuperscript{-1} decreases from 2.4 (Fig. 3a, middle) to 1.4 (Fig. 3a, top). Both bands have contributions from C–N and N–H vibrations of the five-membered ring of the purine (Fig. 1c) including effects by the N9 atom.\textsuperscript{18,41,42}

In contrast, when comparing the spectra of NADH obtained at pH 10 with the spectra obtained at pH 7 (Fig. 3b), fewer differences are found, in agreement with the lack of a deprotonation possibility at the N9 atom of the adenine (Fig. 1b). Nevertheless, some differences are observed, including the shift of the distinctive contribution of the nicotinamide unit that involves the C=C stretching of the nicotinamide ring\textsuperscript{25} which appears at 1636 cm\textsuperscript{-1} at pH 7 (Fig. 3b, middle) and is sharper and found at 1625 cm\textsuperscript{-1} at high pH (Fig. 3b, top). It should be noted here that all spectra were corrected for the spectral contribution from water as a solvent; therefore, no pH-dependent changes in the O–H deformation mode of water are taken into consideration. Furthermore, the band at 1064 cm\textsuperscript{-1} that can be assigned to both the diphosphate as well as the NH\textsubscript{2} rocking vibration of the nicotinamide\textsuperscript{25,34} intensifies (Fig. 3b, top). In the spectrum of FAD (Fig. 3c, top), deprotonation leads to a more pronounced signal at 1373 cm\textsuperscript{-1}, rendering this region very similar to that in the NADH spectrum (Fig. 3b, top). As distinct contribution by the flavin moiety, the band of the NC stretching modes of flavin rings II and III at 1558 cm\textsuperscript{-1}\textsuperscript{28} becomes very strong, probably due to the effects of deprotonation of the N3 atom in ring III, which is expected to take place (Fig. 1a), as the pH chosen here matches the pK\textsubscript{a} of this group.\textsuperscript{48} Moreover, the band of the adenine around 1600 cm\textsuperscript{-1} mainly due to the NH\textsubscript{2} scissoring\textsuperscript{18} becomes visible clearly, and the intensity of the band at 1505 cm\textsuperscript{-1} assigned both to adenine and flavin ring I\textsuperscript{29} decreases. Both can be the effect of different molecular stacking behavior of the molecules when the flavin moiety is deprotonated.

In an acidic environment, at pH 3, adenine is expected to become protonated at the N1 position (Fig. 1c), which has a pK\textsubscript{a} of 4.2.\textsuperscript{46,49} As a consequence, the spectrum of adenine at low pH (Fig. 3a, bottom trace) displays a number of changes. They include disappearance of the very strong
band at 1329 cm\(^{-1}\) that is assigned to CN stretching vibrations in the five-membered ring of adenine,\(^{18}\) the downshift of the frequency of the asymmetric CH deformation mode of the adenine ring from 1364 cm\(^{-1}\) to 1352 cm\(^{-1}\), including a small shoulder at 1340 cm\(^{-1}\) (Fig. 3a, compare middle and bottom), and intensification of the band at 1498 cm\(^{-1}\). The adenine spectrum is in good agreement with the spectrum published previously by Burova et al. and the differences between the N1 protonated and deprotonated forms that were predicted there theoretically.\(^{24}\) Interestingly, it also contains the adenine mode at 1577 cm\(^{-1}\) (Fig. 3a, bottom) that was only visible in the adenine moiety of NADH and FAD at neutral and basic pH (Figs. 3b and 3c, middle and top). Another change in the spectrum of adenine at acidic pH is the upshift of the band at 1600 cm\(^{-1}\) to 1613 cm\(^{-1}\). It can be explained by the close proximity of the protonated N1 atom to the NH\(_2\) group, changing the electron density in the N–H bond of the amino group.

The profile of the spectral region around the strong band at 1332 cm\(^{-1}\) is also changed significantly in the spectra of NADH (Fig. 3b, bottom) and FAD (Fig. 3c, bottom) at low pH, indicating that also here, protonation at the N1 position of the adenine unit plays a major role. Specifically, lowering of the frequency is observed for this and several other adenine ring modes in this region, indicating changes in the environment of the protonated adenine, possibly by different stacking effects. A similar trend was reported for the Raman spectrum of NADH excited at visible wavelength when pH was lowered.\(^{34}\) Different from these data, where the bands at 1308, 1338, 1378, and 1422 cm\(^{-1}\) are replaced by two bands at 1329 cm\(^{-1}\) and 1411 cm\(^{-1}\) at pH 3,\(^{34}\) here, the CH deformation mode\(^{25}\) at 1354 cm\(^{-1}\) is clearly visible, as is the mode at 1305 cm\(^{-1}\), now shifted to 1295 cm\(^{-1}\) (Fig. 3b, compare middle and bottom). The spectrum of protonated NADH also shows a distinct, broad band in the region of the phosphate vibrations with a maximum around 1070 cm\(^{-1}\) that may be indicative of protonation occurring at the phosphate groups (Fig. 1b).\(^3\) In FAD, where pK\(_a\) of the phosphate of 6.2,\(^{50}\) can lead to protonation at the pH chosen here, the same differences were observed.

In the spectrum of FAD at acidic pH, several bands that were discussed above to be specific of vibrations in the flavin moiety become more pronounced, particularly the band at 1160 cm\(^{-1}\), assigned to stretching modes of ring II/III of flavin, or the CC/CN stretching vibrations at 1625 cm\(^{-1}\).\(^{28}\) A main difference between the spectra of FAD and NADH at pH 3 is the presence of the strong adenine band at 1579 cm\(^{-1}\) in the former that has the flavin signal at around 1550 cm\(^{-1}\) as low-frequency shoulder (Fig. 3c, bottom).

**Conclusion**

The UVRR spectra of the previously well-characterized molecules NADH and FAD, as well as of adenine as their common building block obtained at an excitation wavelength of 266 nm at concentrations that are typical of those in cells and tissues were compared. Although at this excitation wavelength the spectra are dominated by contributions from the adenine, differences are found for the two co-factors, due to intramolecular interactions that vary depending on the presence of the flavin or the nicotinamide unit that have been discussed. Moreover, characteristic vibrations of nicotinamide and flavin that appear at similar signal strength as well enable an unequivocal identification of the different species, especially based on vibrational modes that occur at lower frequency. Particularly,
vibrational modes of the isoalloxazine ring system in FAD are observed that enable a distinction from NADH at neutral and basic pH, of relevance for typical bio-environments. Comparison of the pH-dependent spectra showed that the changes cannot exclusively be explained by the protonation or deprotonation of the adenine moiety but also by protonation or deprotonation of other subgroups and changes in the intermolecular interactions when changing the pH. Even though neither the spectrum of NADH nor that of FAD can indicate the strong spectral changes from deprotonation of adenine at N9 position, as the adenine moiety carries a ribose in both molecules, several other differences are found at high pH as well. In FAD, the spectra are indicative of the deprotonation taking place at the N3 position of the flavin. In NADH, vibrations of the nicotinamide moiety are found to vary as well at high pH. At acidic pH, protonation of the adenine unit in the N1 position of the purine is observed and leads to characteristic differences in the vibrational bands of the adenine moiety in both NADH and FAD. On the basis of the knowledge of the pH-dependent spectra, new models of biologically relevant compartments, e.g., mitochondria, can be developed.

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Supplemental material
All supplemental material mentioned in the text, consisting of figures and tables, is available in the online version of the journal.

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