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Orientation and Conformation of NAD and NADH Adsorbed on Colloidal Silver

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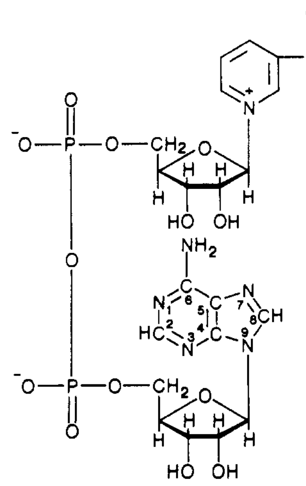
Surface-enhanced Raman scattering (SERS) from NAD and NADH on colloidal silver was observed after the silver particles had been induced to aggregate to varying degrees. The orientation and conformation of the bases, adenine and nicotinamide, in the surface-adsorbed forms of the coenzyme were inferred from the relative intensities of SERS bands. Raman bands assignable to in-plane ring and amino group modes of adenine and δ_s(C–H) of reduced nicotinamide were enhanced the most for NADH on silver at relatively high nucleotide concentrations and in basic media. Alcohol group and ring modes of the sugar, ribose, and pyrophosphate group modes were enhanced to a much lower degree. For NAD, SERS bands assignable to ring breathing modes of nicotinamide and adenine usually dominated the spectra. SERS spectra of nicotinamide, NMNH, NMN, adenine, and imidazole on colloidal silver were used to make more comprehensive band assignments for NAD and NADH on colloidal silver. Indications are that at high nucleotide concentrations NADH binds to the silver surface in a side-on orientation through the N7 and amino group side of adenine and in an end-on fashion to nicotinamide whereas the dominant binding of NAD is to adenine and nicotinamide rings oriented flat on the surface. In these orientations NADH appears to be adsorbed in an extended conformation with the ring systems of both bases assuming tilted configurations with respect to the surface. At low concentrations of NAD and NADH on colloidal silver, band assignments suggest that adenine lies with its plane flat on the surface and that the nicotinamide ring is also nearby so that the coenzymes assume a stacked conformation.

Introduction

Nicotinamide adenine dinucleotide (NAD) and its reduced form, NADH, act as coenzymes in proteins[1-3] that are ubiquitously involved in biological redox processes. They are composed of two 5′-nucleotide bases, AMP and nicotinamide ribose 5′-phosphate, that are linked together by a pyrophosphate bridge. The structure of NAD with its adenine ring atom number scheme is



The conformational flexibility of this linkage in contrast to the structural rigidity of the bases has been shown by NMR spectroscopy[4-10] of NAD and NADH, fluorescence emission[11,12] of NADH, and crystal structure analysis[13,14] of NAD to give both NAD and NADH in solution two main structural forms: an extended one in which adenine and nicotinamide groups lie about 12 Å apart and a folded one in which the bases are stacked parallel to each other at ~3.4 Å apart. The stacked conformer can further adopt either a left- or a right-handed helical structure. Normally, in solution these conformational states are in rapid equilibrium with each other, and the amounts of each depend on conditions of pH, other solvent, and temperature. Particular forms, however, can be trapped by crystallization,[11] binding to enzymes,[1,15] and adsorption at solid interfaces. Very little information is available about the effect of surface adsorption of NAD or NADH on their conformational state. Previous models that have assumed[16] the standard low-energy structure that is generally accepted for nucleotides,[17-19] i.e., the folded conformer of NAD and NADH with adenine lying flat on the surface, can be classified in the realm of wishful thinking rather than fact based on direct experimental evidence.

Surface-enhanced Raman scattering[20] (SERS) of adsorbed molecules on certain conducting metal surfaces lends itself as a spectroscopic tool for determining the orientation[20,21] of planar unsaturated ring systems in monolayer or submonolayer amounts on surfaces. This is particularly timely for the SERS study of NAD and NADH on a silver surface since a considerable body of normal Raman,[22-25] resonance Raman (RR),[26-36] and SERS[37-43]

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spectra of adenine, adenosine, and related nucleotides has already been accumulated. Also, ultraviolet resonance Raman spectra of NADH have been obtained with selective excitation into the individual chromophores of the nicotinamide[44,45] and adenine[45] rings, and SERS spectra of NAD on silver and gold electrodes have been reported[46] at various potentials. Recent studies[47,48] with monooxoanions of the series CrO_4^{2-}, MoO_4^{2-}, and WO_4^{2-} as well as PO_4^{3-} on colloidal silver have indicated the presence of two types of SERS-active sites in aggregates of silver particles: (1) double-sided sites serving as bridges between two neighboring silver particles; (2) single-sided sites with direct coordination to only one particle surface. With the coordination capabilities of NAD and NADH to metal ions, both types of sites could be accommodated. All exposed nitrogen atoms of the adenine ring except from the amino group have been shown[49] to be capable of σ-donor binding to metal ions. In addition, sugar hydroxyl oxygen atoms and phosphate oxygen can coordinate to metals. Nitrogen atoms of the nicotinamide ring are, however, poor donor atoms to metals since the tertiary ring N behaves as a very weak base and the carboxamide group N is also a weak metal binder. It may, however, be possible for the nicotinamide ring to behave as a π-acid ligand[31] to metals such as silver that have been classified as "soft" bases. Nonconjugated olefins such as norbornadiene[50,51] and 1,5-hexadiene[52] and conjugated olefins such as butadiene[53,54] and cyclooctatetraene[55] have been shown to coordinate to "soft" metals through two C=C double bonds.

Experimental Section

Materials. Nicotinamide adenine dinucleotide, free acid (NAD), and β-nicotinamide adenine dinucleotide, reduced form (NADH), disodium salt, Grade III were obtained from Calbiochem and Sigma, respectively. β-Nicotinamide mononucleotide, reduced form (NMNH), sodium salt, NMN, the oxidized form, adenine, adenosine, adenosine 5′-diphosphate (ADP), and imidazole were Sigma grade. Silver nitrate was Alfa Puratronic grade. All other compounds were of the highest available purity and were used without further purification. Deionized double-distilled water was prepared by Corning AG-lb and Ag-3 stills with a Corning 3508-B dimineralizer cartridge.

Sample Preparation. The silver hydrosol with adsorbed citrate was prepared and purified by a modified, Carey Lea procedure.[56] The stock silver sol, prepared by the protocol for sol 1 in previous work,[56] was stored in the dark at a silver concentration of about 2 g/L in a 1.0×10^{-2} M aqueous solution of sodium citrate. This sol consisted mainly of single particles of mean diameter, 13.0 nm. In run 1 aliquots of freshly prepared stock solutions of NAD and NADH in 1 mM aqueous $NaHCO_3$ were added to 10-fold diluted stock silver sol to produce mixtures of pH 8–9, containing 1.3 mM NAD or NADH and 0.2 g/L of silver. When sufficient aggregation of the silver particles had been induced by NAD and NADH (usually after 1–4 days), the mixtures were sometimes further diluted 10-fold with double-distilled water for spectroscopic measurements. In run 2 initial mixtures containing relatively low concentrations (10–100 μM) of NAD or NADH in the deionized[47] silver sol (0.2 g/L of Ag and 3–6 $\mu\Omega^{-1}$ cm^{-1}) at pH 6.5–7.0 were prepared. A sol with 1.2×10^{-4} M NAD and 0.2 g/L of Ag immediately gave a dark red mixture whereas a sol with 1.5×10^{-5} M NAD and 0.2 g/L of Ag gave a purple mixture after several days. Similar procedures were used to obtain silver sols with other adsorbates and 0.2 g/L of Ag.

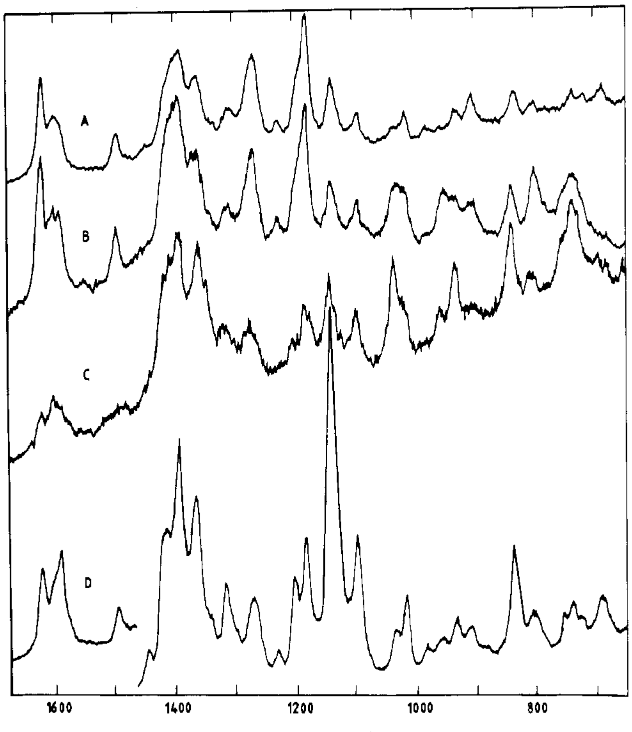

Figure 1. SERS spectra of 1.3 mM NADH (A, B) and NAD (C, 1.3 mM; D, 0.13 mM) on colloidal silver in run 1. Spectra A and C represent mixtures aged for 1 day while spectra B and D were taken after aging for 4 days. Experimental conditions:

	excitation wavelength, nm	incident laser power, mW	spectral slit width, cm^{-1}	photon counting time interval, s
A	576.8	70	5.0	0.080
B	576.8	50	6.0	0.060
C	590.0	80	3.0	0.250
D	647.1	60	5.3	0.200

Tollens' reagent[56] was prepared by mixing 2 mL of 5% aqueous $AgNO_3$ and 1 drop of 10% aqueous NaOH to give a greenish precipitate. Subsequent dropwise addition of 2% aqueous ammonia was continued until the greenish precipitate just dissolved. Test samples of 10 mg of NADH, NAD, or adenosine diphosphoribose (ADPR) in 1 mL of doubly-distilled water were then mixed with the above reagent.

Instrumentation. The Raman instrumentation for CW laser excitation has been previously described.[58]

Results and Discussion

SERS Spectra of NADH and NAD on Colloidal Silver from Run 1. SERS spectra of 1.3 mM NADH and 1.3 mM NAD on colloidal silver, 0.2 g/L, taken after the sol mixture had aged for 1 and 4 days, are shown in Figure 1. Spectrum D in the same figure is for a 10-fold dilution of the NAD on Ag sol that was aged for 4 days. Aging was necessary to obtain a sufficient degree

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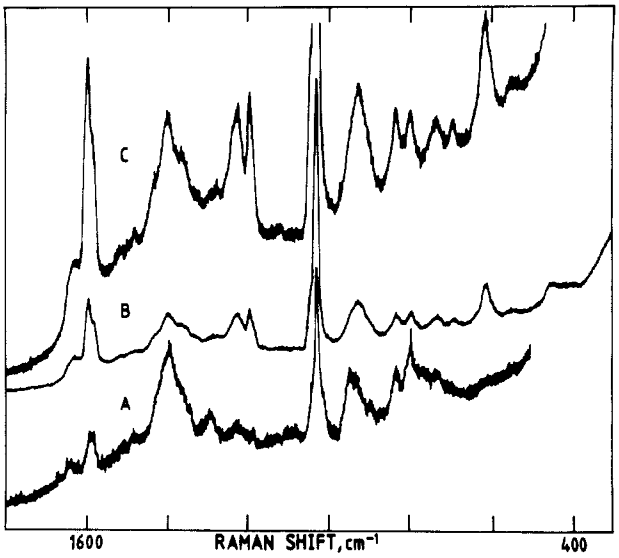

Figure 2. SERS spectra of nicotinamide (A, 20 mM; B and C, 2.0 mM) on colloidal silver. Experimental conditions: 120 mW; 647.1-nm Kr^+; 9.85-cm^{-1} spectral slit width. Counting time interval: (A) 0.400 s; (B) 0.030 s; (C) 0.100 s. Spectra B and C were obtained for the 10-fold diluted Ag sol–nicotinamide mixture.

of aggregation in the silver sols so that SERS spectra could be detected. The wavelengths of excitation from 576 to 647 nm are far away from electronic absorption band maxima[59] of NADH and NAD beginning at 340 and 259 nm, respectively. Also the total concentration level is low enough so that normal Raman scattering was not observed under the same conditions in the absence of silver. Excitation wavelengths were chosen to obtain maximum SERS enhancements that coincided with absorption band maxima[47,56,58] of aggregated silver sols in the 450–650-nm region. Typically our silver sols absorbed maximally between 500 and 600 nm so that laser excitation in the yellow and red regions gave maximum SERS intensity. Similar spectra were obtained with 488.0-nm excitation, but band intensities were often more than 10-fold weaker.

All four SERS spectra in Figure 1 are rich in the number of Raman bands in the 650–1700-cm^{-1} region. Many of the weak Raman bands that have been observed for NAD and NADH in aqueous solutions, especially below 1300 cm^{-1}, have gained in intensity in the SERS spectra. On the whole, though, the SERS spectra of NAD and NADH on colloidal silver are very similar in the positions of band maxima, although relative band intensities do vary considerably. This suggests that only those regions of the coenzyme that are not affected by the oxidation–reduction that is localized in the nicotinamide ring are actually detected in the SERS spectra. Also, in contrast to Raman spectra of NADH and NAD in aqueous solution, the surface Raman spectra of adsorbed NADH and NAD on silver did not show an amide I band characteristic of the carboxamide group in nicotinamide as a sharp intense band at 1686 cm^{-1} in NADH and a broad intense band at 1630 cm^{-1} in NAD. Further, an intense Raman band due to an in-plane stretching mode of the nicotinamide ring occurs at 1540 cm^{-1} for NADH in aqueous solution but was absent or very weak for NADH on colloidal silver. Thus, almost all of the observed SERS bands probably originate from the adenine ring portion of NADH and NAD. Many of these bands have only been observed very weakly before in normal Raman, resonance Raman, or SERS spectra of adenine or adenosine nucleotides.

SERS Spectra of Nicotinamide on Colloidal Silver. To further verify the presence or absence of nicotinamide modes in spectra of Figure 1, we obtained the SERS spectrum of nicotinamide on colloidal silver between 4000 and 300 cm^{-1}, shown in Figure 2 from 1800 to 300 cm^{-1}. Spectrum A was obtained after aging of the silver sol (0.2 g/L)–nicotinamide (20 mM) mixture for 1 day. The mixture had turned very dark red, showing a considerable degree of aggregation, but some residual citrate SERS bands were still detectable particularly at 1400 cm^{-1}. Upon aging for another day the mixture became very dark purple so that to record the B and C spectra in Figure 2 it had to be diluted 10-fold. The SERS intensity of nicotinamide bands in spectra B and C had grown 200-fold over their intensity in spectra A so that citrate SERS bands were no longer observed. The positions (cm^{-1}) of SERS bands and their relative intensities were as follows: 1629 w, 1596 m, 1581 w, sh, 1425 w, sh, 1400 mw, 1365 w, sh, 1224 mw, 1030 vs, 924 mw, 831 w, 795 w, 730 w, 690 w, 610 mw, 455 w. No C–H stretching SERS bands in the 3000–3100-cm^{-1} region were detectable above the climbing shoulder of a bulk water band peaking at 3210 cm^{-1}. Raman spectra of nicotinamide in aqueous buffered solutions have been previously reported.[25] Bands were observed at 1637 w, 1590 s, 1570 w, 1440 mw, 1380 mw, 1200 w, 1150 w, 1050 s, 1030 s, 790 mw, 640 w, and 390 w cm^{-1}. Although the 3-carboxamide pyridine derivative does not possess a tertiary ring nitrogen as in NAD and NADH, it does serve as a model for possible enhanced carboxamide group modes in both NAD and NADH and for enhanced modes of the delocalized ring system of nicotinamide in NAD. One major difference in the SERS spectrum is the presence of a single very intense band for the ring breathing motion at 1030 cm^{-1} instead of a strong doublet (1030, 1050 cm^{-1}) that appears in the solution Raman spectrum[25] of nicotinamide. In Raman spectral studies of adsorbed pyridine this $I_{\nu(i)}/I_{\nu(j)} \sim 0$ ratio has been taken[60] to indicate a strongly chemisorbed species.

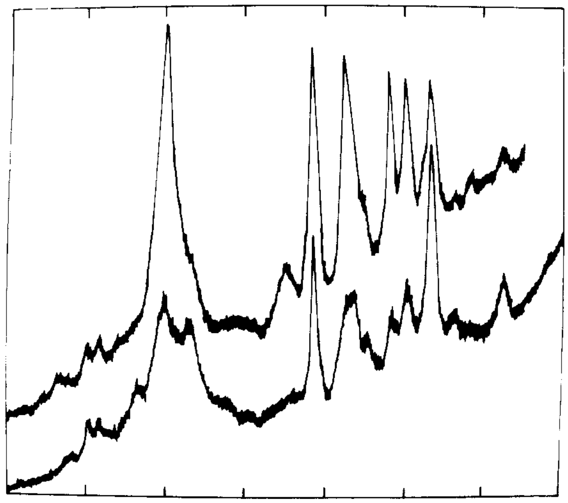

Figure 3. SERS spectra of NAD (top, 0.12 mM; bottom, 15 μM) on colloid silver in run 2. Experimental conditions: 120 mW; 647.1-nm Kr^+; 9.85-cm^{-1} spectral slit width. Counting time interval: (top) 0.240 s; (bottom) 0.220 s.

Relative SERS band intensity arguments support a flat orientation for nicotinamide on the silver particle surface. The single SERS band that was assigned to a ring-breathing mode is at least 3–4 times more intense than any other band in the spectrum. This SERS band intensity pattern is characteristic of adsorbed benzene,[60,61] which cannot lie in a side-on orientation on a silver surface, but it is unlike many of the SERS spectra of pyridine and its derivatives on silver, which generally adopt a side-on configuration on the surface. The low SERS intensity of the band at 1629 cm^{-1}, assignable to the carbonyl stretching mode[24] of the carboxamide group, and other bands assignable to in-plane a_1 modes of the pyridine ring (ν_6 at 620, ν_9 at 1224, and ν_8 at 1596 cm^{-1}) suggests that these modes have only a small radial polarizability component to the surface and, thus, have weak SERS band intensities.[20,21]

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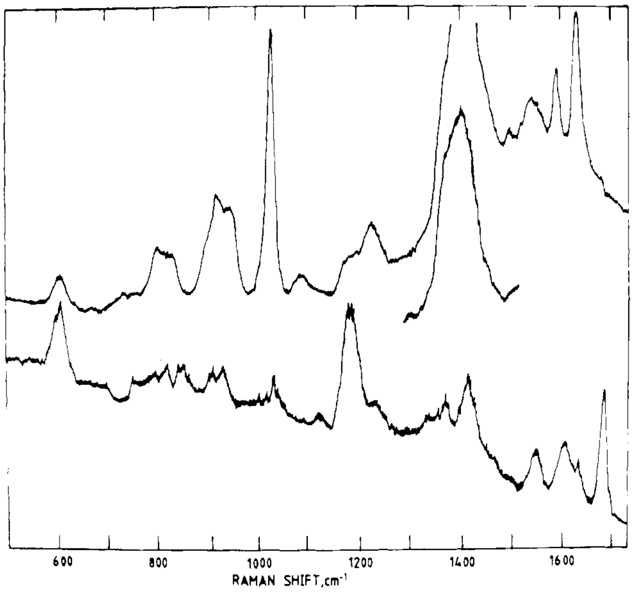


Figure 4. SERS spectrum of 1.3 mM (bottom) and 43 μM (top) NMNH on colloidal silver. Experimental conditions: 50 mW; 488.0-nm Ar^+; 8.0-cm^{-1} spectral slit width; 8K sensitivity. The mixture for the bottom spectrum was freshly prepared; the one for the top spectrum had aged overnight.

SERS Spectra of NAD and NADH on Colloidal Silver from Run 2. The SERS spectrum of the dark red sol with 0.12 mM NAD (Figure 3, top) showed very intense bands characteristic of citrate in the yellow Carey Lea Silver sol[56] as well as one additional major SERS band located at 739 cm^{-1}. The spectrum of the purple sol with 0.15 M NAD (Figure 3, bottom) exhibited citrate bands of much lower intensity, intense SERS bands of adsorbed NAD at 739 and 1030 cm^{-1}, and several other weaker SERS bands of adsorbed NAD at 558, 686, 933, 1338, 1457, 1557, and 1588 cm^{-1}.

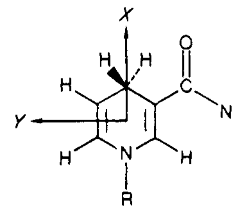
Following the discussion of SERS bands of nicotinamide in the previous section, the intense SERS band of NAD on silver at 1030 cm^{-1} can be assigned to the ring breathing mode of the oxidized nicotinamide ring. The adenine ring breathing mode has been previously identified and can be assigned to the 739-cm^{-1} SERS band of NAD on Ag in Figure 3. Further, similar silver sol–NADH mixtures showed only the intense 739-cm^{-1} band of the adenine ring in addition to citrate bands. In these cases previous considerations of SERS selection rules suggest that the adenine and oxidized nicotinamide rings lie with their planes flat with respect to the silver surface.

SERS Spectra of NMNH and NMN on Colloidal Silver. The SERS spectrum of a freshly prepared mixture of deionized silver sol (3 $\mu\Omega^{-1}$ cm^{-1} and 0.2 g/L Ag) and 1.3 mM NMNH is shown in Figure 4, bottom. Rapid formation of a purple aggregated sol was observed. Several SERS bands that were conspicuously absent in spectra of the NADH–silver sol mixture now occurred intensely. In particular, the amide I mode (mainly ν(C=O)) is found as a very intense SERS band at 1686 cm^{-1}, and a ν(C=C) mode of the reduced nicotinamide ring occurs as a medium band at 1549 cm^{-1}. The positions and relative intensities of NMNH SERS bands between 500 and 1800 cm^{-1} are listed in Table I. Vibrational mode assignments were made by reference to previous assignments[44] for RR bands of NADH and NMNH in solution and orientation and distance considerations for groups in the nicotinamide ring on the silver surface. These are also summarized in Table I. Another very strong SERS band that occurs at 1180 cm^{-1} has a weak counterpart at 1179 cm^{-1} in the solution RR spectrum of NMNH. There is also a strong SERS band at 1180 cm^{-1} for NADH, which must have a significant contribution from a reduced nicotinamide ring mode in NADH. However, NAD on colloidal silver also shows in Figure 1 a medium band at 1180 cm^{-1}, and NADH in solution, when excited with λ_0 = 273.0 nm radiation into an absorption band of the adenine group, gave an RR band[45] at 1175 cm^{-1}. Thus, the 1180-cm^{-1} SERS band of NADH must also be overlapped with an adenine ring mode that occurs with reduced intensity in NAD. The occurrence of intense SERS bands of NMNH on colloidal silver can be explained by reference to the structure of the nicotinamide ring

Table I. SERS and RR Band Positions (cm^{-1}) and Vibrational Mode Assignments for NMNH and NMN

SERS NMNH on Ag sol	RR NMNH in soln[a]	SERS NMN on Ag sol	vib mode
1686 vs	1687		amide I ν(C=O)
1635 w		1635 w	amide I ν(C=O), NMN
1606 m	1614		ν(C=C)
		1590 mw	ν(ring)
1549 m	1548		ν(C=C)
1494 vw			
	1456		amide III ν(C—N)
1410 m	1416		ν(ring)
1371 mw	1378		δ_{as}(CH)
	1302		ν(C—C), ring amide
1231 w	1233	1233 w	ν(C—C) + ν(C—N)
1180 vs	1179		δ_s(C—H)
1122 w			
1030 w		1030 vs	Br, NMN ring
934 mw			
912 w			
853 mw			
822 w			
759 w			
610 s			

[a] Reference 44.



At the relatively high millimolar concentration of NMNH in the mixture with silver sol the molecules will tend to orientate themselves in an edge-on fashion, either end on along the *X* axis or side on in one of the *Y* axis directions, to maximize their coverage on the surface. The end-on configuration is the only orientation that will give high SERS intensities to bands at 1686, 1606, 1549, and 1180 cm^{-1} assignable to the vibrational modes ν(C=O), ν(C=C) in-phase, ν(C=C) out-of-phase, and δ_s(C—H), respectively, all of which will show high polarizability derivative components in the *X* direction, α_{xx}, normal to the surface. This has been shown[20,21] to be a criterion for high intensity in particular SERS bands.

The above mixtures of NMNH and colloidal silver were very sensitive to excitation with blue light and after about 1/2 h of laser excitation at 488.0 nm showed sedimentation of large aggregates of silver particles. At lower concentrations (13 and 43 μM) of NMNH on colloidal silver the mixtures were more stable to sedimentation, but the SERS spectra (Figure 4, top) were complicated by residual bands of citrate, particularly at 1400 and 1030 cm^{-1}, and showed bands that could be assigned to the oxidized derivative, NMN. It was apparent that characteristic strong bands of NMNH at 1686 and 1180 cm^{-1} were absent or occurred very weakly, medium bands were found at 1590, 1549, and 1231 cm^{-1}, a strong band appeared at 1635 cm^{-1}, and the 1030-cm^{-1} band occurred much more intensely than in the SERS spectrum of citrate alone on colloidal silver. For comparison purposes, the SERS spectra of 1.3 mM NMNH (1-week-old solution in 1 mM $NaHCO_3$ freshly mixed with silver sol) and 0.13 mM NMNH (1-week-old mixture with silver sol) taken with 647.1 nm irradiation are shown in Figure 5 (spectra A and B, respectively). The samples were not as light sensitive to excitation in the red. Both SERS spectra now exhibit a very strong band at 1030 cm^{-1} similar to the one for nicotinamide on silver in Figure 2 and, thus, characteristic of the oxidized nicotinamide ring. Spectrum A in Figure 5 still contains medium–weak SERS bands of the reduced

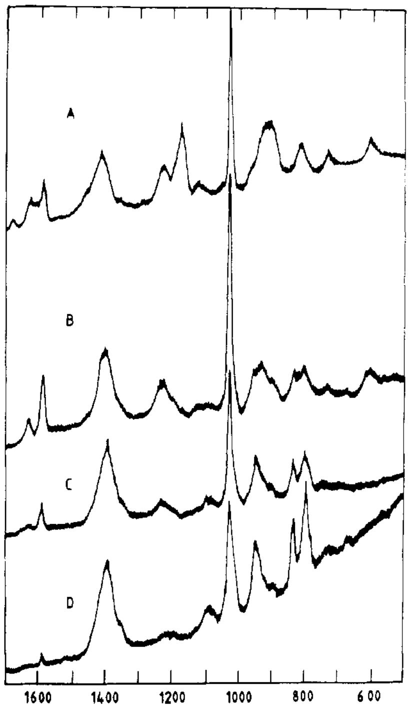


Figure 5. SERS spectra of 1.3 mM NMNH (A, 1-week-old solution in 1 mM $NaHCO_3$), 0.13 mM NMNH (B, 1-week-old mixture with Ag sol), 0.15 mM NMN (C), and 1.5 mM NMN (D) on colloidal silver. Experimental conditions: 80 mW; 647.1-nm Kr^+; 8.0-cm^{-1} spectral slit width; 0.3-s photon counting time interval.

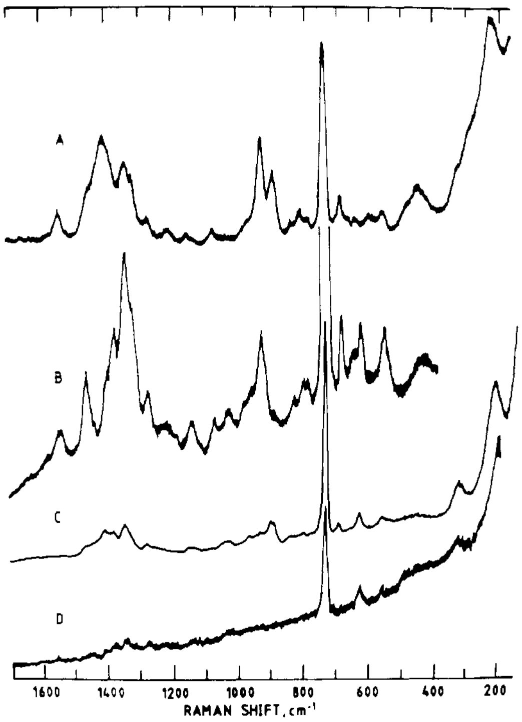


Figure 6. SERS spectra of 10 μM (A, 90 min after mixing), 0.10 mM (B, 3 h after mixing), 0.33 mM (C, 1 h after mixing), and 1.0 mM (D, 3 h after mixing) adenine on colloidal silver. Experimental conditions: 90 mW; 647.1-nm Kr^+; 5.0-cm^{-1} spectral slit width; 0.2-s photon counting time interval.

nicotinamide ring in NMNH at 1180 and 1686 cm^{-1}. Bona fide SERS spectra of 0.15 and 1.5 mM NMN on colloidal silver, overlapped with SERS bands of residual citrate at 1400, 1300, 950, 840, and 800 cm^{-1} are also displayed in Figure 5 (spectra C and D, respectively). The latter spectra confirm the assignment of SERS bands at 1030, 1590, and 1635 cm^{-1} to vibrational modes of the oxidized nicotinamide group as listed in Table I. The strong SERS bands of NMN in partially oxidized NMNH in Figure 4, top spectrum, at 1635 and 1030 cm^{-1}, assignable to amide I and N_{ox} ring breathing modes, respectively, indicate an initial retention of the end-on orientation for the oxidized nicotinamide ring. With elapsed time (1 week in Figure 5B) in NMNH–silver sol mixtures and in NMN on silver the 1635-cm^{-1} SERS band becomes very weak compared to the intense band at 1030 cm^{-1}. Under these latter conditions we believe the N_{ox} ring is oriented flat on the silver surface.

SERS Spectra of Adenine, Adenosine, and Adenosine 5′-Diphosphate (ADP) on Colloidal Silver. Normal Raman spectra of aqueous buffered solutions (pH 6.0) of adenine[22-25] show bands at 1608 mw, 1590 mw, 1478 ms, 1460 w, sh, 1420 w, 1365 m, 1325 s, 1310 w, 1243 s, 1175 w, 1120 m, 1020 mw, 935 m, 893 mw, 800 w, 715 s, 620 m, 580 w, 533 m, 330 ms, and 240 w cm^{-1}. Although SERS spectra of adenine on a silver electrode[41] at several potentials and on colloidal silver[43] obtained by the borohydride reduction method have been reported, we have, for comparison purposes herein, measured spectra for adenine on silver under the conditions of our preparative procedures. In contrast to other adsorbate–silver sol combinations, all mixtures of adenine (10 μM to 1.0 mM) with colloidal silver (0.2 g/L) rapidly aggregated so that spectra had to be obtained within 3–4 h before sedimentation occurred. Also, the 1.0 mM silver sol mixture underwent a sol-to-gel phase transformation after ~3 h. This probably arises from the limited solubility of adenine in water in comparison with other adsorbates that were used and, thus, its marked tendency to adsorb on the solid phase.

Most of the Raman bands of adenine quoted above have their counterparts, albeit at shifted frequencies, in the SERS spectra of adenine on colloidal silver shown in Figure 6. All four spectra, taken for total adenine concentrations of 10 μM, 0.10 mM, 0.33 mM, and 1.0 mM, are dominated by a very strong band at 739 cm^{-1}, and another intense band usually occurs at 1340 cm^{-1}. This type of intensity relationship for the 739- and 1340-cm^{-1} bands was also previously reported[37-43] in most SERS spectra of adenine and its derivatives. The relative intensity of the 739-cm^{-1} band increased with the concentration of adenine for spectra in Figure 6, and in general, the 739-cm^{-1} band was much more intense in our spectra than in previous reports. This behavior is similar to that of nicotinamide on silver and suggests a predominantly flat orientation for the planar adenine ring with respect to the surface throughout the 0.010–1.0 mM concentration range. The same conclusion was reached in a previous study[43] of adenine on colloidal silver.

SERS band frequencies and relative intensities of adenine on silver are collected in Table II. Band assignments were made by reference to previous Raman spectral studies of purine nucleotides and normal mode calculations[22,32,62] for adenine, which, however, have met with much less success for adenine than for the other purine base, guanine.[63,64] It is apparent, though, that in-plane adenine ring (6- and 5-membered) modes (stretching and bending) are observed with medium to medium-weak intensity at about the same positions as in the SERS spectra of NADH and NAD on silver.

Mixtures of adenosine and ADP with the same silver sol under comparable experimental conditions showed very slow aggregation of silver particles and then only at the low concentration end (10 μM) of adsorbate. SERS spectra of 10 μM adenosine on silver showed only a medium band at 738 cm^{-1} and a weak one at 1340 cm^{-1} aside from medium to strong bands of citrate at 800, 838, 900, 940, 1021, and 1400 cm^{-1}. A similar run with ADP on silver was more successful, showing SERS bands at 564 mw, 748 vs,

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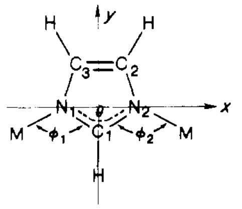
Table II. SERS Band Frequencies (cm^{-1}), Relative Intensities, and Assignments of Adenine on Colloidal Silver

spectrum A, 10 μM	spectrum B, 0.10 mM	spectrum C, 0.33 mM	spectrum D, 1.0 mM	assignt
1538 mw	1538 mw			A ring
1456 w, sh	1456 m	1456 w	1456 w	A ring
1405 s, br		1405 m	1405 w	
	1390 w, sh			
	1369 mw, sh	1369 mw	1369	A ring, δ(C2H)
1340 ms	1340 s	1340 m	1340 w	A ring, ν^5
1324 w, sh	1324 w, sh			
1267 w	1267 mw	1267 w	1267 w	A ring, ν^5 + δ(C8H)
1213 w	1213 w			
1138 w	1138 mw	1138 w	1138 w	A ring, ν(N1C2) + ν(C2N3)
1071 w	1071 w			
	1029 w	1029 w	1029 w	
976 vw, sh	976 w, sh	976 w		
931 s	931 ms	931 w		A ring, δ
895 m	895 vw	895 m		
833 vw	833 w	833 w		
810 w	810 w	810 w		
790 vw	790 w	790 w		
739 vs	739 vs	739 vs	739 vs	A ring, δ^5
690 mw	690 mw	690 w		
643 vw	643 vw			
633 vw	633 mw	633 mw	633 m	
605 w				
562 w	562 mw	562 w	562 w	
488 vw				
457 mw, br	457 mw, br			
333 w, sh		333 m	333 m	
		224 s		

1345 m, 1467 w, 1564 mw, and 1648 w cm^{-1} assignable to adenine ring modes of ADP and medium to weak bands of citrate. SERS spectra were again dominated by a very strong band of the adenine ring at either 738 or 748 cm^{-1}, indicative of a flat orientation for the planar ring on the surface.

SERS Spectra of Imidazole and Histidine on Colloidal Silver. SERS spectra are shown in Figure 7 for two runs of a 1 mM imidazole–silver sol mixture prepared by the standard protocol. In run 1 the spectra were measured 1 day (spectrum C) and 2 days (spectrum D) after preparation. Spectrum E is for the 10-fold diluted mixture from D. In run 2 a similar 1 mM imidazole–silver sol mixture was measured (spectrum B) after 8 days. Also, the SERS spectrum (A) of a 10 μM histidine hydrochloride–silver sol mixture is shown in the same figure.

Under the near-neutral pH conditions and high imidazole concentrations in the imidazole–silver sol mixtures, we expect imidazole to stand up on its side on the silver surface and assume an ionized imidazolate anion form that is capable of coordination to two silver particle surfaces in a bridging fashion to favor the aggregation that is a necessary condition for observation of SERS. The SERS spectral data appear to support this contention. The crystal and molecular structure of the blue polymorph[65,66] of bis(imidazolato)copper(II), CuIm$_2$, has been shown by X-ray analysis to contain imidazolate ion as a bidentate ligand, bridging Cu(II) atoms in an infinite, 3-dimensional lattice network. The geometric constraints on the bridging structure



are such that a single imidazolate cannot serve as a bidentate ligand to a single metal atom. Infrared and Raman data[67-70] for imidazole and its derivatives have suggested the following mode assignments[71] in C_{2v} symmetry for CuIm$_2$:

A$_1$		B$_2$	
1544	ν(ring)	1497	ν(ring)
1265	ν(C=C)	1433, 1419	ν(ring)
1177, 1164	ν(ring)	1340, 1329	δ(C—H)
1095	δ(C—H)	1074	δ(C—H)
956, 943	δ(ring)	924	δ(ring)

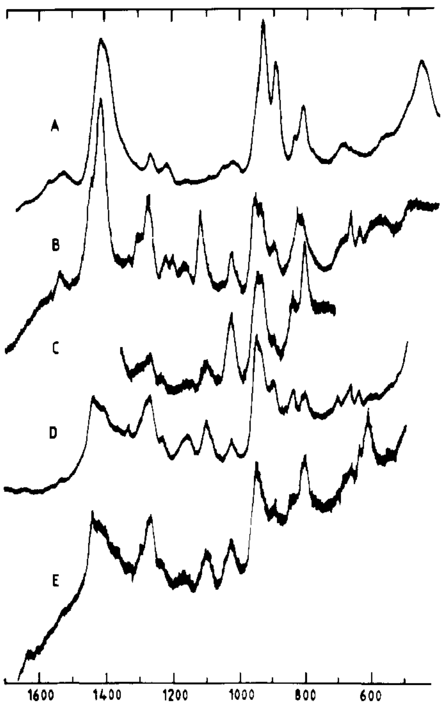


Figure 7. SERS spectra of 3.3 μM L-His·HCl (A) and 1.0 mM imidazole (B–E) on colloidal silver. Experimental conditions: 647.1-nm Kr$^+$; spectral slit width, 8.0 (A, B), 10.0 cm^{-1} (C–E); laser power, 80 (A, B, E), 90 mW (C, D). Photon counting time interval: (A) 0.04 s; (B) 0.19 s; (C) 0.1 s; (D) 0.1 s; (E) 0.23 s.

The above mode assignments were thus used as a guide for interpreting the imidazole on silver SERS spectra. Spectrum C in Figure 7 shows the appearance of additional relatively weak bands besides those of citrate adsorbed on silver. As aggregation of the silver colloid, induced by the addition of imidazole, progressed further, these extra bands grew significantly more intense and are shown in Figure 7D after an elapsed time of 2 days. The most intense SERS band at 949 cm^{-1} is associated with the symmetrical in-plane ring deformation mode, which occurred at 956 cm^{-1} in CuIm$_2$. The next most intense bands of adsorbed imidazole are located at 1267 and 1100 cm^{-1} and are assigned to symmetrical ring stretching and C–H deformation modes, respectively. These modes are expected to have a large polarizability component normal to the silver surface if the imidazolate is orientated in an edge-on fashion with its x axis (N···N line) perpendicular to the surface. Thus, N (imidazole) coordination to surface silver atoms can give both single-sided and double-sided or bridging coordination in direct analogy to the bridging role of imidazole in the CuIm$_2$ structure in the solid state. Similar Raman bands of single-crystals of CuIm$_2$ at 1265 and 1095 cm^{-1} were assigned to symmetrical vibrational modes of imidazolate, which

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Table III. Positions (cm^{-1}), Relative Intensities, and Assignments of SERS Bands of Imidazole and Histidine on Colloidal Silver

spectrum A	spectrum B	spectrum C	spectrum D	spectrum E	predominant mode assignt
1564 w					
1526 w	1536 w				
	1436 w, sh		1436 s	1436 s	ν(ring), im
1410 vs					ν(COO$^-$), his
	1405 vs		1400 m, sh	1400 m, sh	ν(CO$^-$), cit^{3-}
			1331 w		
	1300 w, sh				
1267 mw	1269 m	1267 w	1267 s	1267 s	ν(ring), im
		1231 vw	1231 w		
1219 mw	1220 w				
	1200 w				
	1167 w	1156 vw	1156 mw	1156 w	ν(ring), im
	1117 m	1100 w	1100 m	1100 m	δ(C–H), im
1052 w					
1026 w	1021 mw	1026 s	1026 mw	1026 m	ν(C–C), cit^{3-}
	952 m	941 s	941 vs	941 s	δ(ring, im + ν(C—C), cit^{3-}
932 vs	936 m				
894 s	895 w	895 w	895 w	895 w	ν(C—COO$^-$), his + δ(ring), im
840 w	826 m	838 w	838 mw	838 w	cit^{3-}
814 m	812 m	805 s	805 mw	805 ms	cit^{3-}
700 mw, br	700 w		705 w		
	671 mw		671 mw	671 w	π(ring), im
	648 w		644 w	644 w	π(ring), im
				614 ms	π(ring), im
567 w					
460 s, br					

possessed a large α_{xx} component. The reasons for the intense 949-cm^{-1} band are not as clear since it was most intense in CuIm$_2$ crystal spectra for an orientation in which both α_{xx} and α_{yy} components were large. Some overlap with a 936-cm^{-1} band of adsorbed citrate could raise its relative intensity as is also apparent for the 1436-cm^{-1} band of imidazole and the 1400-cm^{-1} band of residual citrate. The 1156-cm^{-1} band can be linked with its 1177-, 1164-cm^{-1} counterpart in CuIm$_2$, which was assigned to the symmetrical ring breathing mode. Other ring stretching at 1544 and 1331 cm^{-1} and out-of-plane modes between 600 and 700 cm^{-1} are all relatively weak.

The δ(CNC) in-phase bending motion perpendicular to the 2-fold symmetry axis as part of the overall ring deformation mode will give a large α_{xx} polarizability change. This coupled with some mixing with the ring breathing mode will produce an intense SERS band when imidazole is orientated edge-on the surface. The ring breathing mode of imidazolate at 1177, 1164 cm^{-1} in CuIm$_2$ is much more intense in the α_{yy}-dominated spectrum than in the α_{xx}- or α_{zz}-dominated spectra. Thus, it is not one of the most intense bands in the SERS spectra.

Further support for these assignments was gained from the SERS spectrum of L-His on colloidal silver in Figure 7A. A very strong band assignable to an in-plane ring deformation mode of the imidazole group in L-His occurs at 932 cm^{-1}. Also, a medium–weak band assignable to symmetrical ring stretching was located at 1267 cm^{-1}. SERS bands near these positions were observed weakly at 928 cm^{-1} or not at all for the other amino acids[72] (L-Met, L-Asp, L-Lys, L-Cys, L-cystine) on colloidal silver. Due to structural changes, the 1100-cm^{-1} SERS band (δ_s(C–H)) of imidazole on silver has no counterpart in the spectrum of L-His on silver.

A 10-fold dilution of the 1 mM imidazole–silver sol mixture after the second day of equilibration gave the SERS spectrum E in Figure 7. Citrate bands at 1400, 1030, and 805 cm^{-1} now feature more prominantly in the spectrum, and a very weak out-of-plane deformation at 614 cm^{-1} in spectrum D appears very intensely in spectrum E. Also, the 1267-cm^{-1} band has intensified. This appears to reflect the presence of bands that have a z component in their polarizability, i.e., α_{zz} and α_{xz} or α_{yz}. The band at 1265 cm^{-1} in CuIm$_2$ had its greatest relative intensity when α_{zz} was the dominant polarizability component being measured. Assignment of vibrational modes in the SERS spectra of imidazole on colloidal silver are summarized in Table III.

These spectra support the contention[20,21] that vibrational modes with a large z component (z axis normal to the surface) in their polarizability should give rise to intense SERS bands. Thus, at high imidazole concentrations when in-plane modes of imidazole gave the most intense SERS bands, imidazole is expected to be oriented edge-on the surface so that α_{xx}- and α_{yy}-polarized bands dominate the spectrum. On the other hand, at lower imidazole concentrations, it appears that out-of-plane Raman bands and those that are α_{zz}-polarized are the most intense. For this to occur, the imidazole ring should lie with its plane flat on the surface.

Imidazole Ring Modes in Adenine Nucleotides. Some of the more prominent SERS bands of NADH and NAD on colloidal silver in Figures 1 and 3 were identified with the 5-membered imidazole ring portion of adenine by reference to the SERS spectra of imidazole and histidine on silver and the solution and polarized single-crystal Raman spectra[71] of bis(imidazolato)copper(II). The most intense polarized Raman bands of CuIm$_2$ in aqueous solution could be assigned to in-plane, totally symmetric modes of the imidazolate ring at 1435, 1338, 1263, and 1185 cm^{-1}. SERS spectra of imidazole on silver in a side-on orientation showed similar strong bands at 1436 and 1267 cm^{-1} but a medium–weak band at 1156 cm^{-1} and a weak band at 1331 cm^{-1}. Surface spectra of adenine in a flat orientation on silver gave three bands in similar positions, 1456 m, 1340 s, and 1267 mw, but no detectable band in the 1150–1200-cm^{-1} range. However, an 1180-cm^{-1} band has also been identified in UV resonance Raman studies[36] of purines as the ring breathing mode of the 5-membered ring. Medium to strong SERS bands were observed at 1268 and 1180 cm^{-1} for both NADH and NAD on silver in Figure 1, whereas only weak to very weak bands were located at 1446 and 1332 cm^{-1}. These latter bands can now be assigned to in-plane modes localized in the 5-membered ring of adenine.

The in-plane ring deformation modes of imidazole in CuIm$_2$ were identified at 956, 943, and 924 cm^{-1} from infrared and Raman data[67-70] for imidazole and its derivatives. Very strong SERS bands of imidazole on silver at 941 cm^{-1} and of the imidazole ring in histidine on silver at 932 cm^{-1} are assigned to a δ(ring) mode of the imidazole ring oriented side on to the silver surface. A weak to strong SERS band of adenine on silver at 931 cm^{-1} is assignable to a similar δ(ring) mode in the 5-membered ring of adenine. The very strong SERS band of adenine on silver

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(72) Curley, D.; Siiman, O. *Langmuir*, in press.

at 739 cm^{-1} does not have a counterpart in spectra of imidazole oriented side on to the silver surface; however, polarized Raman spectra of $CuIm_2$ single crystals showed an intense band at 736 cm^{-1} that could be identified with a ring deformation mode with a dominant *xz* component of the imidazolate ring. Further, resonance Raman spectra[33] of dAMP and their analysis showed that bands at 1339 and 730 cm^{-1} were assignable to modes localized in the 5-membered ring of adenine. Thus, the 1338- and 739-cm^{-1} SERS bands detected for NAD on Ag in Figure 3 and the 734-cm^{-1} band that occurs strongly in some spectra in Figure 1 can also be assigned to modes localized in the imidazole portion of adenine.

Six-Membered Ring Modes in Adenine Nucleotides. The NH_2 scissors mode has not been observed intensely in Raman spectra of adenine nucleotides nor is it expected in the SERS spectra of adenine lying flat on the silver surface. An adenine mode, NH_2 deformation + $\nu(C6-NH_2)$, observed weakly at 1670 cm^{-1} in the Raman spectrum of adenine[25,62] and intensely by us at 1648 cm^{-1} in the FT-IR spectrum of a 0.3% potassium bromide pellet of NADH, was not detected in the solution normal Raman spectra of NADH or NAD. The two medium to weak SERS bands at 1590 and 1600 cm^{-1} in Figure 1 may be assigned to the triene systems, C2=N3—C6=C5—N7=C8, in purines and is probably related to the ν_{8a} ring mode of substituted benzenes. The identical position of SERS bands at 1618 and 1493 cm^{-1} for NADH and NAD on silver in Figure 1 suggests that these bands do not originate from amide group modes in the nicotinamide ring, which undergoes a chemical change, but rather from the amino group modes of adenine, which is unchanged. The intense 1618-cm^{-1} SERS band of NADH on silver, which does not appear in solution Raman spectra of NADH, is then assigned to the deformation mode of the amino group. The relatively large downward shift of this band from 1648 cm^{-1} indicates direct interaction of the amino group with the silver surface. Another SERS band that is found with medium-weak intensity when the 1618-cm^{-1} band occurs strongly is located at 1493 cm^{-1} and can be assigned to the $\nu(C6-NH_2)$ stretching mode. When an 1137-cm^{-1} SERS band is observed as the most intense one in Figure 1, spectrum D, bands of the six-membered adenine ring at 1590 and 1600 cm^{-1} in NAD also show their maximum intensity. We note that 1,3,5-triazabenzene adsorbed on silver shows[61] an intense SERS band at 1125 cm^{-1}, concurrently with strong bands at 1575 and 1555 cm^{-1}, and SERS spectra of adenine on silver have a medium–weak band at 1137 cm^{-1}. The 1137-cm^{-1} SERS band of NAD and NADH on colloidal silver is thus assigned to an in-plane $\nu(N1C2N3)$ mode. Both NAD and NADH in aqueous solution and adenine on silver show other intense Raman bands in the 1420–1300-cm^{-1} region that have counterparts in the SERS spectra in Figures 1 and 3. Further vibrational band assignments, supported by ab initio SCF MO calculations[62] of the vibrational frequencies and modes of adenine and detailed Raman studies of adenine nucleotides, are summarized in Table IV.

Ribose and Pyrophosphate Group Vibrations. Pyrophosphate and phosphodiester Raman bands that have been identified in NAD and NADH occur at ~1100 cm^{-1} (1080 and 1110 cm^{-1}), $\nu_s(PO_2)$, and at 800 cm^{-1}, $\nu_s(P-O-P)$. The intensity ratio of these two Raman bands, $I_{\nu(POP)}/I_{\nu(PO_2)}$, has been used[73,74] as an indicator of the ordered structure about the pyrophosphate linkage in ribonucleotides and AMP. In our surface spectra of NAD and NADH on silver, there is a SERS band at 1095 cm^{-1} in all cases, which can be assigned to $\nu_s(PO_2)$; however, either no band intensity or a very weak intensity is observed in the 850–900-cm^{-1} region. As in previous cases we interpret this $I_{\nu(POP)}/I_{\nu(PO_2)} = 0$ ratio to mean that there is disorder in the rotational conformational states of the pyrophosphate bridge for NAD and NADH adsorbed on the colloidal silver surface.

Table IV. SERS Band Frequencies (cm^{-1}), Relative Intensities, and Assignments of NADH and NAD on Colloidal Silver

NADH		NAD		
spectrum A	spectrum B	spectrum C	spectrum D	assignt
1618 s	1618 s	1618 w	1618 m	A ring, NH_2(sc)
1600 w	1600 w	1600 mw	1600 sh	A ring, ν^6
1590 sh	1590 w	1590 sh	1590 m	N_{ox}, ν(ring)
	1546 vw	1546 vw		A ring
1493 mw	1493 mw	1493 vw	1493 mw	A ring, $\nu(C6-NH_2)$
1446 vw	1446 vw	1446 vw	1446 w	
1416 sh	1416 sh	1416 vw	1416 w	A ring, ν^6
1393 s	1393 vs	1393 vs	1393 s	cit^{3-} + A ring
1363 mw	1363 mw	1363 s	1363 ms	A ring, $\delta(C2H)$
1332 sh	1332 sh	1332 sh	1332 sh	A ring, ν^5
1313 w	1313 w	1313 w	1313 mw	A ring, NH_2 rock
1268 ms	1268 ms	1268 mw	1268 mw	A ring, $\nu^5 + \delta(C8H)$
1225 w	1225 w	1225 vw	1225 w	A ring
1200 sh	1200 sh	1200 w	1200 w	
1180 vs	1180 s	1180 mw	1180 m	N_{red}, $\delta_s(C-H)$ + A ring, Br^5
1137 m	1137 m	1137 m	1137 vs	A ring, $\nu(N1C2) + \nu(C2N3)$
1090 w	1090 w	1090 mw	1090 m	P, $\nu_s(PO_2^-)$
1029 sh	1029 m	1029 s	1029 sh	N_{ox}, Br
1012 mw	1012 sh	1012 sh	1012 mw	R, $\nu(C-O) + \nu(C-C)$
980 w	980 vw	980 vw	980 w	
954 vw	954 mw	954 w	954 w	R, $\nu(C-C) + \nu(C-O)$
929 w	929 vw	929 m	929 mw	R, $\nu(C-C)$ + A ring, δ^5
904 mw	904 w	904 wv	904 w	
	880 vw		880 vw	
837 mw	837 mw	837 ms	837 m	P
800 w	800 m	800	800 w	RP
780 vw	780 vw		780 vw	
754 sh, vw	754 sh, vw	754 sh, vw	754 w	
734 w	734 m	734 s	734 mw	A ring, δ^5
690 w		690 w	690 mw	A ring
641 w				
600 w				
536 ms				A ring
485 mw				RP
456 m				A ring

Ribose sugar group Raman bands in adenosine nucleotides have previously been assigned to weak to very weak bands in the 900–1100-cm^{-1} region. Alcohol group $\nu(C-O)$ modes and sugar ring $\nu(C-C)$ modes occur in this region and can be SERS enhanced, as seen in our previous work[56,75] with citrate ion adsorbed on colloidal silver, if the ribose group lies in the proximity of the surface. These bands appear to be particularly intense in spectra B and C of Figure 1 at the same time that the band at 734 cm^{-1} due to the adenine ring breathing mode reaches its maximum intensity. As in guanine, the latter mode appears to be sensitive to the conformation of the ribose ring and the torsional angle about the glycosidic link between adenine and ribose. The side bands that appear at 757 and 725 cm^{-1}, particularly in spectrum D of Figure 1, probably indicate the presence of both C-2′ endo and C-3′ endo adenine–riboside sugar conformations, that have been shown to exist for NAD complexed[14] to Li(I) and NAD bound[76] to some enzymes, respectively. In contrast, the bottom SERS spectrum in Figure 3 shows a very narrow single band at 739 cm^{-1}, which indicates a single dominant conformation for NAD in this case.

Structure of NAD and NADH on Colloidal Silver. Two most probable explanations exist for the absence of many SERS bands of the nicotinamide group in most spectra in Figure 1: (1) the nicotinamide ring is too far away from the surface to have its vibrational modes enhanced significantly; (2) the orientation of

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the nicotinamide ring with respect to the surface is such that its usually intense modes are absent or very weak according to surface Raman selection rules. As we have seen for nicotinamide, NAD, and NMN on colloidal silver in spectra in Figures 2, 3, and 5, the latter situation can arise for some SERS bands if the oxidized nicotinamide ring as well as its carboxamide substituent were to lie flat with respect to the surface. However, the nicotinamide ring breathing mode should then still be observed as an intense SERS band at 1030 cm^{-1}. This is not always the case for NAD on colloidal silver in spectra C and D of Figure 1 since in one spectrum a medium–strong band is observed at 1029 cm^{-1} but in the other spectrum only a weak shoulder appears at 1029 cm^{-1}. Also, the same band at 1029 cm^{-1} is found weakly in the SERS spectra (A and B, Fig. 1) of adsorbed NADH, which indicates some conversion of NADH to NAD during the 1–4-day equilibration period. Further, a decrease in the intensity of the strong 1180-cm^{-1} SERS band and increase in band intensity at ~1400 cm^{-1} of NADH on silver in going from spectrum A to B in Figure 1 is consistent with the assignment of the band to the δ_s(C–H) mode of the reduced nicotinamide ring since NMNH spectra in Figure 4 show a similar trend in the process of oxidation to NMN. Also, the absence of a SERS band of the amide group at 1686 cm^{-1} may not be diagnostic of the absence of the reduced nicotinamide ring in NADH from the silver surface. For partially oxidized NMNH on silver in Figure 5A, the SERS band at 1686 cm^{-1} is very weak; nevertheless, the 1180-cm^{-1} band is still relatively strong. A strong band at 1590 cm^{-1} in Figure 1D for NAD on silver might also be assigned to ν(ring) of the oxidized nicotinamide ring as in spectra of NMN in Figure 5. Since the 1029-cm^{-1} band of N_{ox} is weak and the 1635-cm^{-1} band is absent in this spectrum of NAD on silver, a side-on orientation of N_{ox} on the surface may be implied. Therefore, we prefer the second explanation for the absence of many SERS bands of the nicotinamide ring at relatively high nucleotide concentrations and in the pH 8–9 range.

Previous SERS spectra[46] of 2 mM NAD on a silver electrode (0 to −1.0 V vs SCE) showed two intense bands at 735 and 1335 cm^{-1}, assignable to adenine ring modes. Other weak bands were observed in the 600–1600-cm^{-1} region but were not well resolved. For 2 mM NAD on a gold electrode (−0.25 to −1.0 V vs SCE) the SERS spectra were more potential dependent, and other bands, particularly one at 1035 cm^{-1} assignable to the nicotinamide ring breathing mode, intensified and became clearly resolved. The overall structural picture, though, is one in which either adenine alone or both adenine and nicotinamide are orientated with their planes flat on the surface so that only certain ring modes that possess a large polarizability component normal to the surface dominate the SERS spectra. Weak, submonolayer adsorption of NAD on the electrode surfaces at the potentials that were used is probably most responsible for this flat orientation.

Similar behavior in most of the SERS spectra of adenine and its various derivatives on a silver electrode was reported by other investigators.[37–42] Notable differences were observed[41] for SERS spectra of 5 mM adenosine or AMP on a silver electrode, particularly at the more positive potential of +0.02 V vs SCE. In these two cases other medium to strong SERS bands were also observed for the adsorbed adenine ring at 1120, 1161, 1242, 1309, 1327, 1382, 1419, 1456, and 1479 cm^{-1} in the 1100–1500-cm^{-1} region. Also, in the SERS spectrum[42] of poly-A on a silver electrode at −0.1 V vs SCE, two strong bands were reported at 1327 and 731 cm^{-1} while medium bands were clearly located at 1464, 1379, 1245, 1131, and 1095 cm^{-1}. Adenine on colloidal silver also showed[43] very strong SERS bands at 739 and 1339 cm^{-1} as well as a medium band at 1454 cm^{-1} and other weak bands at 1145 and 1265 cm^{-1}. Thus, variable intensity in SERS bands of the adenine ring under different conditions appears to indicate several orientations for the ring with respect to the silver surface.

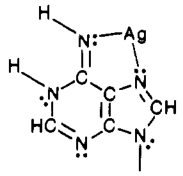
X-ray crystal structures[49] of metal ion complexes with adenosine phosphates have shown three sites for metal coordination: (1) phosphate oxygen; (2) sugar (ribose) alcohol oxygen; and (3) adenine ring nitrogen. All three sites are also prime candidates for direct chemisorption to silver on the particle surface. Structural information on NADH and NAD has been mainly obtained from an X-ray analysis[14] of a Li(I) salt of NAD in the solid state and from high-resolution NMR studies[4–10] of both NAD and NADH in solution. In solution, two folded over conformations with adenine and nicotinamide rings stacked parallel to each other at a distance of 3.4 Å are believed to exist in rapid equilibrium with an extended conformation with the two bases about 12–13 Å apart. In the crystalline state an extended conformation of NAD was found with Li(I) coordinated to pyrophosphate oxygen and adenine N7. Also, for purine nucleotides, in general, metal–heterocyclic nitrogen binding at N7 is preferred[49] over N1 or N3 with metal ions such as Cd^{2+}, Co^{2+}, Ni^{2+}, Pt^{2+}, Cu^{2+}, and Zn^{2+}.

At high nucleotide concentrations, the relatively large SERS enhancement of bands in the 1100–1620-cm^{-1} region for both NAD and NADH that have been identified with in-plane adenine ring vibrational modes, implies an end-on or side-on orientation of the adenine ring with respect to the silver surface, so that in-plane vibrational modes will have large polarizability derivatives[20,21] normal to the silver surface. In spectra A and B (NADH on Ag) the SERS bands of adenine at 1268 and 1618 cm^{-1} are intense. At the same time, the band due to the ring deformation mode at 734 cm^{-1} is relatively weak in both spectra. On the other hand, in spectrum D (NAD on Ag) a SERS band at 1137 cm^{-1} is the most intense one and the δ^5 adenine ring mode shows only a weak band at 734 cm^{-1}. This intense 1137-cm^{-1} SERS band of NAD on Ag was assigned to an in-plane mode of the adenine ring. It is not, however, the usual, intense Raman band of adenine or its derivatives either in aqueous solution or on silver surfaces that have been investigated thus far. A counterpart may have been observed in the SERS spectrum[41] of AMP on a silver electrode at 1120 cm^{-1}.

On the basis of these band assignments, its appears that the most intense SERS bands of NADH on colloidal silver are those that are diagnostic of an end-on orientation of N_{red} on silver or are consistently involved in vibrational motion with N7 or atoms (C6, NH_2, C5, C8) on the same side of the adenine ring as N7. The other side of adenine consisting of N1, C2H, and N3 gives SERS bands of considerably lower intensity in spectra A and B of Figure 1. Thus, we conclude that the adenine group in NADH at high concentrations binds in a side-on orientation through N7 and the amino group to the colloidal silver surface. This is the usual coordination site of adenine for metals.

The most intense SERS bands of NAD at high concentrations on colloidal silver are different from those of NADH under similar conditions. In spectrum C of Figure 1 strong bands due to ring modes of nicotinamide and adenine at 1029 and 734 cm^{-1}, respectively, show that most NAD molecules are orientated with their bases flat with respect to the surface. Another marked change is the relatively large intensity of a SERS band at 1137 cm^{-1}, especially in spectrum D of Figure 1, which is associated with the in-plane N1C2N3 stretching mode. This supports a side-on orientation through N3 to the silver surface for the majority of NAD molecules. This is not the usual coordination site of adenine for metals, but it has been shown[49] to be the preferred mode of coordination of K(I) to the adenine ring in ADP in the crystalline state. There is some flexibility in the tilting of adenine with respect to the surface such that various modes of vibration can have their greatest polarizability component normal to the surface and thus experience a large SERS intensification. This can produce the observed variation in relative SERS band intensities for NAD and NADH between 1100 and 1620 cm^{-1} shown in spectra A–D in Fig. 1. A weak 1029-cm^{-1} band and a medium band at 1590 cm^{-1} in Figure 1D may indicate a side-on or end-on orientation for N_{ox} on silver though this should be accompanied by intense amide group bands.

SERS experiments with the adsorption of adenine and adenosine nucleotides on other silver surfaces[37–43] have shown that Raman bands at 739 and 1339 cm^{-1} are the most intense, and thus, adenine lies flat[43] on the silver surface. Conditions on electrodes surfaces such as the coadsorption of the supporting electrolyte, chloride ions, electrode potential, and the formation of Ag(I) complexes on the silver surface appear to favor[41] the latter orientation of adenine. Our SERS spectra[47] of CrO_4^{2-}, MoO_4^{2-}, and WO_4^{2-} on the same colloidal silver particles as used in the present experiments have shown that these anions are not adsorbed as Ag(I) complexes on colloidal silver. Thus, we do not expect formation of Ag(I)–NAD or Ag(I)–NADH complexes on the colloidal silver surface. In any case we have checked the possibility of Ag(I) interactions with NADH and NAD since Ag(I) is known[77] to interact strongly with "soft" bases such as the isoalloxazine ring of flavins in their oxidized state. Freshly prepared Tollens' reagent was mixed with the respective pyridine nucleotides. After several hours, mixtures of the reagent with NADH had turned to a light red color and showed scattering from colloidal silver particles in a Tyndall beam. Mixtures with NAD turned dark red but showed no scattering from colloidal silver. We also found that mixing ADPR with the reagent gave, almost instantly, the same dark red solution, absorbing maximally at 430 nm. Since no reduction of Ag(I) is observed with NAD or ADPR, formation of the red solution species appears to require a chemical reaction localized in the adenine portion of the nucleotides. This probably involves conversion of adenine to its normally minor imino tautomer,[3] which can be stabilized in ammoniacal silver(I) solutions by formation of a Ag(I) chelate coordinating to N7 and the imino nitrogen atom, depicted as follows:



Fractionation of the dark red mixtures formed from mixing $[Ag(NH_3)_2]OH$ with NADH and aging for 1 day was carried out by liquid chromatography on Bio-Gel P2 (100–1800-Da range). An initial fraction with a strong electronic absorption band at 430 nm and weaker bands at 205 and 260 nm was separated. The strong 340-nm band of NADH was absent, suggesting oxidation to NAD and reduction of Ag(I) to elemental silver, as shown by the appearance of colloidal particles. The 40-fold molar excess of Ag(I) over NADH in the Tollens' reagent tests was sufficient to also convert the NAD to the red product. No resonance Raman spectra of the red species were detected with 457.9-nm Ar^+ excitation even though the absorbance at 458 nm was 0.60 compared to 0.66 at the absorption band maximum. The SERS spectra of NAD and NADH in Figures 1 and 3 do not contain any unusual bands that might be associated with chemically modified species of adenine; therefore, we conclude that a similar surface Ag(I)–NAD or Ag(I)–NADH chemical interaction is not occuring in our silver sol systems.

It appears most likely that the two ring systems in surface-adsorbed NAD at low concentrations and near pH 7 adopt a folded-over conformation in which the rings are stacked. If NAD were to bridge pairs of silver particles in the aggregates that it induced to form in double-sided coordination (i.e., adenine flat on one surface; nicotinamide flat on the other surface), then we would expect the SERS band intensity ratio, I_{1030}/I_{739}, to remain about the same as the 2:1 ratio observed in solution Raman spectra of NAD. On the other hand, in single-sided coordination of NAD to a silver particle in the aggregates with adenine closest to the silver surface and nicotinamide lying 3.4 Å away and an $(a/r)^{12}$ dependence of SERS intensity on distance[71,72] from the surface, a 1.8-fold lower intensity is predicted for SERS bands of the nicotinamide ring. The latter situation is what appears to be observed in Figure 3, bottom spectrum, where $I_{1030}/I_{739} = 0.86$, and in Figure 1C. The alternative extended conformation for NAD in this case is unlikely for two reasons: (1) the nicotinamide ring will be located at ~13 Å from the adenine ring so that single-sided coordination through adenine will give SERS bands of nicotinamide an 8-fold lower intensity and thus be very weak; (2) double-side coordination with both rings flat on their respective surfaces may not be possible since X-ray data for NAD show that the two rings are nearly perpendicular to each other in the extended conformation. The above considerations have assumed a homogeneous mode of NAD coordination to the surface. Heterogeneity in the surface structure of NAD, such as some molecules adsorbed with adenine flat on the surface and others absorbed with nicotinamide flat on the surface, could also account for the observed SERS spectra. In an extended conformation there is flexibility in the pyrophosphate linkage to allow a variety of side-on orientations for both adenine and nicotinamide rings with respect to the surface. It appears that these side-on orientations of the two bases are different for NADH and NAD; i.e., oxidation of the nicotinamide ring induces a change in orientation for not only its ring system but also that of the adenine ring.

Available X-ray data[76] on coenzyme (NAD) binding to proteins (glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase) show that in each case NAD is in an extended conformation in which the pyrophosphate group is directed away from the two bases and is exposed to binding to the protein. Also, the planes of the nicotinamides and adenine rings located at 13 Å apart are not parallel to each other but are very nearly perpendicular. This conformation of NAD binding to proteins is also very appealing for its structure on colloidal silver as deduced from SERS spectra in run 1. With adenine and ribose groups in closest proximity to the surface, the nicotinamide ring would lie far enough away (~13 Å) such that, with an $(a/r)^{12}$ dependence on distance[78,79] from the surface for SERS intensity, its Raman bands would be observed very weakly. With primary silver particles of diameter 13 nm, or $a = 6.5$ nm, and a 0.2-nm separation between adenine and silver for direct adsorption on the surface, we estimate an 8.4-fold lower SERS intensity for Raman bands of the nicotinamide group at a distance of 1.3 nm from the adenine group.

Thus, in many ways NAD or NADH anchored onto the surface of small silver particles has the potential to behave as an artificial enzyme, if the adenine moiety simply functions as a group to tie down the coenzyme either in a hydrophobic pocket having minimal interaction with amino acid residues of an enzyme or onto a surface. The nicotinamide group is then left exposed near the surface but not chemisorbed to the surface of silver particles so that it can react with specific substrates. Of course, in competitive binding experiments of NAD or NADH for enzyme or silver particle surface, the presence of the latter would compete very effectively for the same binding site(s) on the coenzyme and thus disrupt the usual biological redox processes. Also, in polynucleotides containing adenine, silver surface binding to both N7 and N3 sides of adenine and/or to the π-delocalized, planar adenine ring in a flat orientation will tend to disrupt the helical structure. This will be true of base pairing between the amino group hydrogen and N7 in the interior of the poly-A helix with the N3 side left exposed on the surface as well as for base pairing to adenine sites in nucleic acids.

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Registry No. NAD, 53-84-9; NADH, 58-68-4; NMNH, 4229-56-5; NMN, 1094-61-7; Ag, 7440-22-4; nicotinamide, 98-92-0; adenine, 73-24-5; imidazole, 288-32-4.

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