A Raman and Infrared Spectroscopic Study of the 3-Carbonyl Group of Pyridine Nucleotide Coenzymes and Related Model Compounds†

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ABSTRACT: The carbonyl frequencies exhibited in the Raman spectra of nicotinamide, 3-acetylpyridine, and nicotinaldehyde were observed as a function of solvent. In non-hydrogen-bonding solvents, similar carbonyl frequencies (~1690–1700 cm^{-1}) were observed for these three compounds. In hydrogen-bonding solvents (H_2O, D_2O, CD_3OD), the carbonyl frequency of nicotinamide decreased markedly (~30–40 cm^{-1}), while that of the other compounds remained essentially unchanged. These results were interpreted in terms of preferential stabilization of a resonance form of the carboxamide group, $-(O^-)C{=}NH_2^+$, by hydrogen-bonding solvents resulting in increased single-bond character (decreased vibrational frequency) for the carbonyl of the carboxamide substituent. Similar results were observed with the *N*-methyl derivatives of these compounds, leading to the suggestion that the proposed resonance effect was independent of the nature of the N substituent and thus operative in NAD^+. The result would be that the carboxamide group of NAD^+ in aqueous solutions would have an appreciably more polar nature than suggested by the conventional representation, $-C(=O)NH_2$. In contrast to the results with the oxidized pyridine derivative, NADH and its analogs showed no solvent effect on the carbonyl frequency in the Raman spectra. Thus the polar character suggested for the 3-carboxamide group of the oxidized compounds may be considerably diminished as a result of reduction of the ring. These results are interpreted as an indication of pronounced differences between the oxidized and reduced compounds with regard to the electronic structure of the carboxamide group and its interaction with its solvent environment and with the pyridine ring. Such differences may be relevant to an understanding of the nature of the interactions between the pyridine coenzymes and enzymes.

Interaction between the adenine and pyridine rings of pyridine nucleotide coenzymes has been detected by a variety of techniques including fluorescence (Weber, 1957; Shifrin and Kaplan, 1960), nuclear magnetic resonance (nmr) (Jardetzky and Wade-Jardetzky, 1966; Sarma *et al.*, 1968; Catterall *et al.*, 1969; Sarma and Kaplan, 1970a,b; Blumenstein and Raftery, 1972, 1973), and circular dichroism (Miles and Urry, 1968). Although it has been pointed out (Jacobus, 1971) that the available data do not permit an unequivocal assignment of structures for the coenzymes, a stacking of the aromatic ring systems seems to be favored by most of these authors. The parallel arrangement of the rings appears to be maintained largely by hydrophobic interactions between the π-electron systems. Additional intramolecular interactions include those between the positively charged nitrogen of the pyridine ring in the oxidized coenzyme and the negatively charged oxygen of the diphosphate backbone, and also between the amino group of the adenine moiety and the carboxamide carbon on the pyridine portion (Blumenstein and Raftery, 1972, 1973).

On the basis of their nmr studies, Sarma *et al.* (1968) explicitly excluded hydrogen bonding as being of importance in the interaction between the adenine and pyridine moieties; a similar conclusion was drawn by Catterall *et al.* (1969) on the basis of structural considerations. The initial intention of the present study was to utilize an alternative technique, Raman spectroscopy, as the means for detecting hydrogen bonding to the carboxamide group of the pyridine moiety. Our results support the conclusion of the earlier authors, *i.e.*, the Raman spectra do not indicate intramolecular hydrogen bonding between the adenine and pyridine moieties. In addition, however, our results suggest that the electronic structure of the carbonyl of the carboxamide group in *the oxidized coenzymes* is considerably different from that of a "normal" carbonyl, with important contributions from the resonance form, $-(O^-)C{=}N^+H_2$, to the electronic structure.[1] The net result is to confer appreciably greater polar character on the carboxamide group than would be expected on the basis of its conventional representation, $-C(=O)NH_2$. The carboxamide of the *reduced* coenzyme does not appear to share the polar character of the carboxamide on the oxidized form, *i.e.*, its electronic structure appears to be more adequately described by the conventional representation. These may be important considerations when seeking the mode of interaction of pyridine nucleotide coenzymes with enzymes.

Experimental Section

Materials. Nicotinaldehyde, nipecotamide (3-piperidinecarboxamide), and 3-acetylpyridine were purchased from Aldrich Chemical Co. NAD^+, NADH, NMNH, nicotinamide, and *N*-methylnicotinamide iodide were obtained from Sigma Chemical Co., and the 3-acetyl analogs of NADH and NAD^+ from P-L Biochemicals. Methyl iodide, dimethyl sulfoxide, and acetonitrile were products of J. T. Baker Co. Deuterium oxide was purchased from Columbia Organic Chemicals, *p*-dioxane from Matheson, Coleman & Bell, and methyl alcohol-d_4 from British Oxygen Co. The dimethyl sulfoxide was distilled over 4

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[1] This type of resonance has frequently been cited as a factor in determining the planarity of the amide (peptide) bond of proteins (*e.g.*, Hanlon, 1970). The potential significance of the contribution of this resonance to the electronic properties of the carboxamide group of pyridine nucleotides does not appear to have been recognized explicitly, however.

Å molecular seives before use; all other solvents were used without purification.

The *N*-methyl derivatives of nicotinaldehyde and 3-acetylpyridine were prepared by methylation with methyl iodide (Böger *et al.*, 1967). The melting points for the *N*-methyl derivatives were 174–175 and 163–165°, respectively, in excellent agreement with the values previously reported for these compounds (Böger *et al.*, 1967; Ginsburg and Wilson, 1957).

Methods. Raman spectra were obtained with an instrument assembled from components including a Spex 1400 double monochromator, RCA C 31034 photomultiplier tube (S20 response), Victoreen VTE-1 dc amplifier, and a Hewlett-Packard Moseley 7100 B chart recorder. The lasers used were a Spectra-Physics Model 164 argon ion laser (maximum output 1.6 W at 5145 Å) or a Spectra-Physics Model 165 krypton ion laser (maximum output 0.76 W at 6471 Å), powered by a Spectra-Physics Model 265 exciter. Samples were placed in 4-mm o.d. L-shaped glass tubes and the cell was then sealed. The laser beam was focused onto the samples with a lens (10-cm focal length) and Raman scattering was collected at 90° to the incident light. The geometrical arrangement utilized transverse illumination by the laser beam and transverse observation of the scattered light.

Spectra were routinely recorded at room temperature (20°). When more precise temperature control was desired, the sample tube was inserted into an unsilvered Dewar through which N_2 gas at the desired temperature was passed. Temperature in the sample chamber was monitored with a copper-constantan thermocouple and a Leeds and Northrup potentiometer, and could be maintained within ±0.2° of a selected value.

Infrared spectra were recorded with a Perkin-Elmer 225 instrument. Aqueous solutions were run in 0.2-mm thick cells with IRTRAN windows, and nonaqueous solutions in NaCl cells with 0.103-mm sample thickness. The vibrational frequencies reported are considered accurate to ±2 cm^{-1}.

Results and Discussion

Typical Raman spectra for several of the compounds used in this study are shown in Figure 1.

Carbonyl Frequency in Simple Pyridine Derivatives. Table I lists the carbonyl amide I (Hanlon, 1970) frequencies observed in the Raman spectra of nicotinamide, 3-acetylpyridine, and nicotinaldehyde, dissolved in various solvents.[2] The frequencies for nicotinamide were confirmed by infrared spectroscopy. The carbonyl frequencies of the 3-acetyl and 3-aldehyde compounds were essentially insensitive to solvent, showing little difference in the values observed in solvents as different as water and dioxane. In contrast, the carbonyl frequency of nicotinamide was markedly lowered when this compound was dissolved in D_2O or CD_3OD; in the other solvents, the carbonyl frequency of nicotinamide was quite similar to that observed with the other compounds. From the solvent properties given in Table I, it is clear that this marked lowering of the carbonyl frequency of nicotinamide in CD_3OD or D_2O is not related to the dipole moment or dielectric constant of the solvent. The possible exchange of amide hydrogens ($-NH_2 \rightleftarrows -ND_2$) would not appreciably affect the amide I vibration (Hanlon, 1970). Thus, it seems likely that the pronounced effect of CD_3OD and D_2O is related to the hydrogen-bonding capability of these solvents.

[2] It is, of course, an approximation to assign a vibrational frequency to a single functional group. However, detailed normal coordinate analysis indicates that the C—O force constant provides the major portion of the potential energy distribution for the vibrational frequency observed in this range.

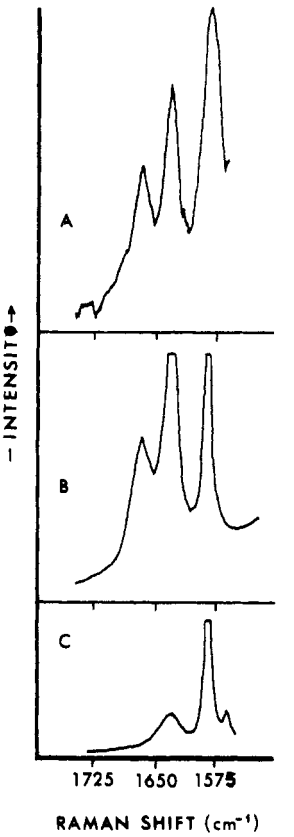


FIGURE 1: Typical Raman spectra in the carbonyl stretching region of several compounds used in this study: concentration, 0.25 M in D_2O; temperature, 19°; excitation wavelength, 514.5 nm; (A) NAD^+; (B) *N*-methylnicotinamide; (C) nicotinamide.

The aromatic ring is not a requirement for this marked sensitivity of the carbonyl frequency to solvent. The carbonyl frequencies observed in Raman spectra of the fully saturated analog, nipecotamide, were 1623 and 1672 cm^{-1} in D_2O and dimethyl sulfoxide, respectively. The corresponding values observed in infrared spectra were in excellent agreement, being 1621 and 1674 cm^{-1}, respectively.

The effect of solvent on the carbonyl frequency in the Raman spectrum of nicotamide was further investigated in experiments employing D_2O-dioxane mixtures (Table II). A marked shift of the carbonyl peak to lower frequency, along with significant broadening of the band, was observed as the D_2O content of the solvent was increased (Figure 2).

The above experiments indicate that the marked solvent effect was concomitant with the presence of an amino group adjacent to the absorbing carbonyl. In terms of the adjacent amino group, one can write the following resonance structures

$$\underset{\text{I}}{\text{RC}(=\text{O})\text{NH}_2} \longleftrightarrow \underset{\text{II}}{\text{RC}(-\text{O}^-)=\text{NH}_2^+}$$

The resonance effect would tend to increase the C—O distance in the carbonyl group and decrease the force constant, and thus the vibrational frequency. As a result of the expected increased interaction (*via* hydrogen bonding) with resonance form II, hydrogen bonding solvents could increase the contribution of this form to the overall electronic properties of the carbonyl group. This could provide an explanation for the marked lowering of the carbonyl frequency of the carboxamide group in hydrogen-bonding solvents. In non-hydrogen-bonding solvents, form II would be expected to make a lesser contribution to the electronic structure of the carbonyl, and the observed frequency

TABLE I: Effect of Solvent on Carbonyl Frequencies in Raman Spectra of Nicotinamide, 3-Acetylpyridine, and Nicotinaldehyde.

Solvent	Carbonyl Frequency (cm^{-1})			Solvent Properties	
	3-Carboxamide[b]	3-Aldehyde	3-Acetyl	Dipole Moment[c]	Dielectric Constant[d]
None[a]	1675	1700	1688		
D_2O	1637 (1633)	1705	1684	1.88	78.25
H_2O		1703	1685	1.92	78.54
CD_3OD	1658 (1654)	1711	1692	2.97[e]	32.63[e]
CH_3CN	1689 (1690)	1707	1692	3.39	38.8
Dimethyl sulfoxide	1686 (1687)	1702	1688	3.9	48.9
Dioxane	1691 (1690)	1707	1692	0	2.2
Benzene	(1690)			0	2.28
$CHCl_3$	(1690)			1.55	4.81

[a] Pure compounds; solution concentrations are 0.25 M. [b] Numbers in parentheses are carbonyl frequencies observed in infrared spectra. [c] Values from McClellan (1963). [d] Values from Jacob *et al.* (1971) and Handbook of Chemistry and Physics (1972–1973), Chemical Rubber Co., Cleveland, Ohio. [e] Values for methanol.

TABLE II: Raman Carbonyl Frequency for 0.25 M Nicotinamide in Dioxane–D_2O Mixtures.

Vol % Dioxane	Carbonyl Frequency (cm^{-1})	Width at Half-Height (cm^{-1})[a]
100	1691	11
94	1665	27
88	1659	27
75	1658	25
50	1648	37
25	1639	32
0	1637	32

[a] Error limits ±10%.

should be more nearly that of a "normal" unperturbed carbonyl.

Carbonyl Frequency in N-Methylpyridine Derivatives. The carbonyl frequencies for the *N*-methyl derivatives of nicotinamide, nicotinaldehyde, and 3-acetylpyridine are given in Table III, and compared with the values observed for the corresponding unmethylated compounds. N-Methylation results in a slight (~10–20 cm^{-1}) increase in the carbonyl frequency. Again, the carboxamide derivative (*N*-methylnicotinamide)

TABLE III: Raman Carbonyl Frequencies for *N*-Methyl Derivatives of Nicotinamide, 3-Acetylpyridine, and Nicotinaldehyde.

Solvent[a]	Carbonyl Frequency (cm^{-1})		
	3-Carboxamide	3-Aldehyde	3-Acetyl
D_2O	1667 (1637)[c]	1720 (1705)	1704 (1684)
Dimethyl sulfoxide	1696 (1686)	1715 (1702)	1702 (1688)
None[b]	1680 (1675)	1699 (1700)	1702 (1688)

[a] 0.25 M solutions of the iodide salts were prepared in either D_2O or dimethyl sulfoxide. [b] Pure compounds. [c] Values in parentheses are for the corresponding unmethylated compound; these values are given in Table I, but repeated here for ease of comparison.

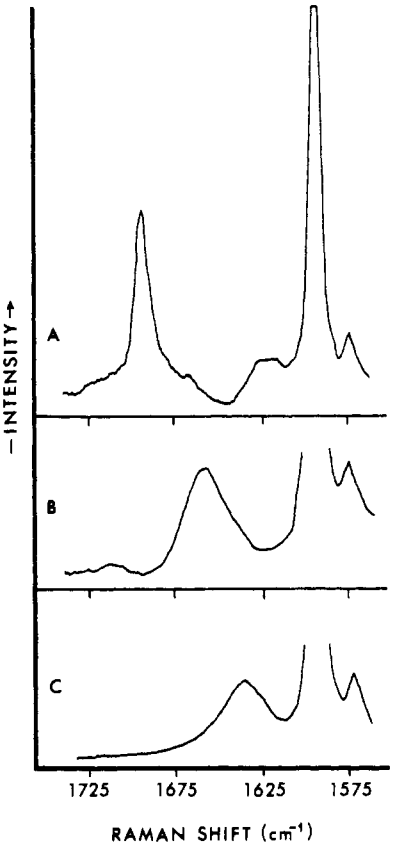


FIGURE 2: Raman spectra in the carbonyl region of 0.25 M nicotinamide in D_2O–*p*-dioxane solutions of varying volume per cent: (A) 0% D_2O; (B) 25% D_2O; (C) 100% D_2O.

shows a marked lowering of the carbonyl frequency in the hydrogen-bonding solvent, D_2O; the other compounds are essentially insensitive to changing the solvent from dimethyl sulfoxide to D_2O. Thus, quaternization had a relatively minor influence on the observed carbonyl frequency and on the solvent effect.

The slight *increase* in carbonyl frequency observed to result from N-methylation (Table III) suggests that the electrons of the carbonyl group have become more localized, resulting in an increased double-bond character. This is opposite to the effect one might expect from quaternization of the ring nitrogen, and

TABLE IV: Effect of Temperature on Carbonyl Frequencies in Raman Spectra of NAD^+, NADH, and Related Compounds.

Compound[a]	Solvent	Carbonyl Frequency (cm^{-1}) at 19°	70°
NAD^+	D_2O	1667[b]	1667
3-Acetyl analog of NAD^+	D_2O	1708	
3-Aldehyde analog of NAD^+	D_2O	1717	1717
N-Methylnicotinaldehyde	D_2O	1720	1720
NMNH (reduced nicotinamide mononucleotide)	D_2O	1689	1689
NADH	D_2O	1689	1689
NADPH	H_2O	1690	
NADH	H_2O	1689	1689
NADH	Dimethyl sulfoxide	1689	1689
3-Acetyl analog of NADH	D_2O	1679	
3-Acetyl analog of NADH	Dimethyl sulfoxide	1679	

[a] 0.25 M solutions were prepared in the indicated solvent. [b] In agreement with a value of 1666 cm^{-1} observed by infrared spectroscopy.

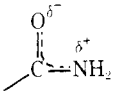
the explanation is not immediately obvious.[3] In any case, these results certainly provide no indication that the positive charge on the pyridine ring results in any increased delocalization of the electrons from the carbonyl group in the aromatic ring.

Carbonyl Frequency in NAD, NADH, and Analogs. The carbonyl frequencies for NAD, NADH, and various related compounds in water and dimethyl sulfoxide solutions are given in Table IV. The spectra were recorded at both low (19°) and elevated temperature (70°). The existence of an intramolecular hydrogen bond in the "stacked" conformation of the coenzyme should result in a lowering of the carbonyl frequency (Vinogradov and Linnell, 1971). Thus, if such an intramolecular hydrogen bond were present, the carbonyl frequency should be higher at 70°, a temperature at which the intramolecular complex is largely disrupted (Miles and Urry, 1968). It is evident that temperature has *no* effect on the observed carbonyl frequency. Thus it may be concluded, in agreement with previous investigators (Sarma *et al.*, 1968; Catterall *et al.*, 1969), that there is no hydrogen-bonding interaction between the carbonyl of the 3-carboxamide group and the adenine ring. The identical carbonyl frequencies observed for NAD^+ and the model compound, *N*-methylnicotinamide, which contains no adenine moiety, confirm this conclusion.

There is excellent agreement between the carbonyl frequencies observed for NAD^+ and for the 3-aldehyde and 3-acetyl analogs of NAD^+ (Table IV) and the carbonyl frequencies noted for the corresponding *N*-methylpyridine derivatives (Table III). This suggests little or no interaction between the carbonyl group of the 3-substituent and the substituent on the nitrogen of the pyridine ring, *i.e.*, the properties of the 3-substituent are essentially independent of the nature of the *N*-substituent. Thus, we suggest that the resonance interaction proposed to exist in the model compounds is also operational in the more complex NAD^+. This may *not* be the case for the reduced compounds, however, in which the carbonyl frequencies show no solvent effect (Table IV).

Conclusion

Sarma and Kaplan (1970b) have stated, "It is difficult to generalize why the different analogs substituted in the three position of the pyridine moiety react so differently with the various pyridine nucleotide dehydrogenases. The variation is certainly not attributable to difference in the stacking of the bases. The possibility exists that different enzymes can distinguish changes in the side chain at the three position." Thus, the electronic distribution in the amide group of NAD^+ and NADH could be of major importance in determining interaction at the binding sites for the coenzymes. The present investigation provides evidence in support of the view that, in aqueous solutions, the three position $-C(=O)NH_2$ group of NAD^+ may be more adequately represented by the structure



However, this structure does *not* appear to make an equally significant contribution to the electronic structure of NADH. Thus, changes in oxidation state of the ring affect not only the ring itself, but also the electronic properties of the 3-carboxamide group. These differences are likely to be of great significance in determining enzyme–coenzyme interactions.

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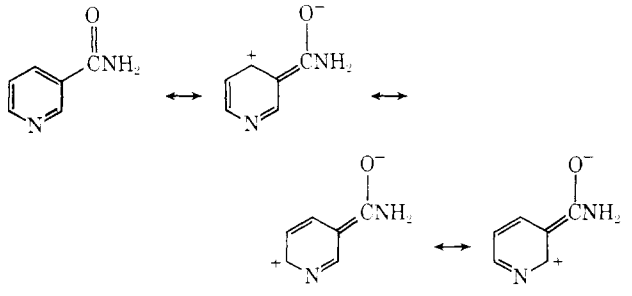
[3] One possibility would be that resonance forms represented as



might make a significant contribution to the electronic structure of the unquaternized compound. Quaternization, with the resulting positive charge conferred on the ring, should then drastically reduce the contribution of these forms, thereby increasing the "double-bond" character of the C—O bond and raising the frequency.

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Intramolecular Hemiacetal Formation in 8-Formylriboflavine†

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ABSTRACT: Reports in the literature suggest that the flavine site of *Chromatium* cytochrome c_{552} contains a covalent adduct in which a cysteine residue is linked to 8-formyl-FAD in a thiohemiacetal linkage. This indication of a biological function for 8-formylflavines and the lack of published information on their chemical and physical properties prompted the present study of three 8-formylflavine analogs. It was found that a ribityl hydroxyl group forms a stable hemiacetal with the 8-formyl group in 8-formylriboflavine. Acid hydrolysis of 8-formylriboflavine converts it from a species which gives a negative 2,4-dinitrophenylhydrazine test to one which gives a positive test. On standing in aqueous solution it reverts to the original species. 8-Formyltetraacetylriboflavine and 8-formyl-3-methyllumiflavine have hydroquinone absorption spectra characterized by maximal absorption at 520 and 392 nm, while reduced 8-formylriboflavine has little or no absorption in this spectral region. All three flavine analogs give identical absorption spectra when overreduced with $TiCl_3$ in 6 N HCl. The oxidation-reduction potential of 8-formylriboflavine is −0.159 V, which is raised after acid hydrolysis to −0.090 V. The respective oxidation-reduction potentials of 8-formyltetraacetylriboflavine and 8-formyl-3-methyllumiflavine are −0.006 and −0.045 V. The circular dichroism spectrum of 8-formylriboflavine is drastically altered with respect to riboflavine, whereas the spectrum of 8-formyltetraacetylriboflavine is very similar to that of tetraacetylriboflavine. Studies with molecular models suggest that the 5′-hydroxyl group is involved in hemiacetal formation.

Studies on the covalently bound flavine of *Chromatium* cytochrome c_{552} (Hendricks *et al.*, 1972, Kenney *et al.*, 1972, 1973) strongly suggest that the flavine (FAD) is bound by way of a thiohemiacetal linkage to a cysteine residue in the polypeptide chain. Since the flavine component of this adduct is 8-formyl-FAD, a biological role is established for 8-formylflavines. Although 8-formylflavines have been synthesized and used as intermediates in the syntheses of other 8α-substituted flavines (Salach *et al.*, 1972; McCormick, 1970), there is little or no information in the literature on their chemical and physical properties. Information of this kind is of value for further studies of the *Chromatium* flavine.

This paper reports a comparison of the physical and chemical properties of three 8-formylflavine analogs. The results show that in the case of 8-fRF,[1] a side-chain hydroxyl group (most likely that in the 5′ position) forms a hemiacetal with the 8-formyl group. This intramolecular interaction profoundly affects the oxidationdshreduction potential and circular dichroic (CD) properties of the 8-formyl-substituted isoalloxazine ring.

Experimental Section

Flavine Analogs. 8-FRF was synthesized by acid hydrolysis (6 N HCl, reflux for 2 hr) of 8α-dibromotetraacetylriboflavine. The dibromo compound was prepared by prolonged heating of tetraacetylriboflavine with excess bromine in the presence of dibenzoyl peroxide (Ghisla *et al.*, 1970). 8-fRF was also synthesized according to the procedure outlined by McCormick (1970). In the early part of this work, the samples of 8-fRF used were gifts from Dr. G. Blankenhorn, University of California, Davis, Calif., and from Dr. Peter Hemmerich, University of Konstanz, Germany.

8-Formyl-3-methyllumiflavine was synthesized as described by Salach *et al.* (1972). The sample of 3-methyllumiflavine used in this synthesis was a gift from Dr. S. Ghisla, the University of Michigan. 8-fAc_4RF was observed to be a degradation product of 8α-cysteinyltetraacetylriboflavine upon prolonged storage of the thioflavine in aqueous solution (1 week or longer). Purification of the formylflavine involved preparative high-voltage electrophoresis (pH 1.6) and subsequent descending paper chromatography in solvent A (see below).

Homogeneity of all flavine analogs was monitored by thin-layer chromatography on cellulose plates (Eastman Kodak 13255), using 1-butanol-acetic acid-H_2O, either 4:2:2, v/v (solvent A) or 4:1:5, v/v (upper layer) (solvent B), for development.

Methods. Anaerobic spectrophotometric titrations with dithionite solutions were performed under helium in a glass titration cell slightly modified from the design of Burleigh *et al.*

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[1] Abbreviations used are: 8-fRF, 8-formylriboflavine; 8-fAc_4RF, 8-formyltetraacetylriboflavine.