Aquacobalamin (Vitamin B_{12a}) Does Not Bind NO in Aqueous Solution. Nitrite Impurities Account for Observed Reaction

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Numerous papers in recent years have claimed evidence for the binding of NO to aquacobalamin (vitamin B_{12a}) in aqueous solution according to the overall reaction given in eq 1.[1−8] This

$$B_{12}-H_2O + NO \leftrightarrow B_{12}-NO + H_2O \qquad (1)$$

reaction has been interpreted to play an important role in various biological processes,[1−3] and typical studies include the modification of the biological action of nitric oxide in vivo (in particular, the inhibition of NO activity in some pathological stages resulting in an overproduction of NO)[3] and the identification of the exact nature of the neurotransmitter released in certain tissues by the so-called "nitrergic nerves".[4−7] The latter studies are based on a comparison of the inhibitory effects observed in the presence of aquacobalamin on the relaxation produced by NO and some NO-donating compounds, on the one hand, and nerve stimulation, on the other hand. Despite these claims, it was reported 30 years ago that aquacobalamin does not undergo a substitution reaction with NO as shown in reaction 1.[9] This apparent discrepancy has now been reinvestigated using modern spectrophotometric and electrochemical techniques.

We have developed a general interest in the interaction of NO with metal complexes in an effort to clarify the mechanism of its biological and environmental role.[10−14] During these studies we noticed that it is practically impossible to prepare weakly acidic aqueous solutions of NO that are totally free of nitrite. There are several explanations that can account for this: NO in commercial gas cylinders degrades as a function of time, such that it is essential to use freshly filled cylinders; the contamination mainly consists of N_2O and NO_2, which reacts with NO to produce N_2O_3 and in turn decomposes to NO_2^- and NO^+ in aqueous solution; the reaction of NO with oxygen in the aqueous phase leads to the formation of NO_2^-,[15,16] and the presence of traces of oxygen in the setup used for the preparation of aqueous NO solutions can lead to considerable contamination with nitrite.[16] Therefore, the preparation and handling of solutions of NO must be performed under strictly inert atmospheres, especially in cases where a reaction with nitrite may affect the observed results and lead to erroneous conclusions. It has been reported[9] that nitrite binds rapidly to aquacobalamin with a binding constant of $2.3 \times 10^5\ M^{-1}$, which could therefore account for the apparent discrepancy mentioned above.

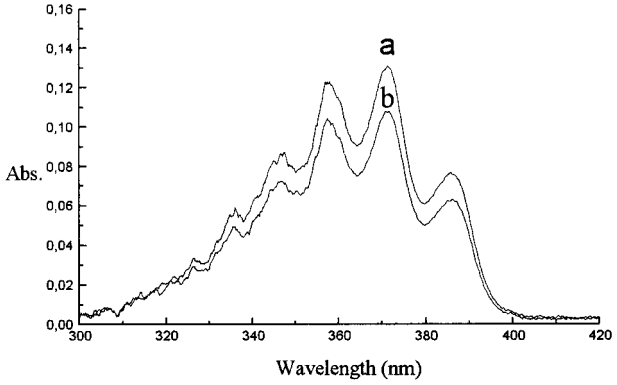

Figure 1. UV−vis spectra recorded for deoxygenated 0.01 M $HClO_4$ (pH 2) saturated with NO (a) or containing 2×10^{-3} M $NaNO_2$ (b).

Table 1. NO_2^- Concentration in NO-Saturated Acetate Buffer Solution (pH 4.5, $I = 0.5$ M, Room Temperature): Comparison between Electrochemical and Spectroscopic Measurements

NO electrode measurement[a]			
NO (mM)	$NO + NO_2^-$ (mM)	NO_2^- (mM)	spectroscopic measurement[a] NO_2^- (mM)
1.3 ± 0.1	3.4 ± 0.3	2.1 ± 0.4	2.3 ± 0.3

[a] Mean values of at least three measurements.

In our experiments the NO concentration in solution was monitored with a very sensitive NO electrode.[17] The determination of NO with this amperometric sensor is based on its diffusion through the gas permeable membrane and subsequent electrochemical oxidation inside the electrode. The dependence of the resulting current on the NO concentration was found to be linear in the concentration range 0−800 μM. More concentrated solutions were appropriately diluted with deoxygenated water. The nitrite present in aqueous solution could be determined either from the characteristic fingerprint spectrum of HONO in the range

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300−390 nm recorded after acidification of the NO solution to pH 2 (at which 94% of nitrite ions are protonated, since the pK_a value of HONO is 3.2[18]) or by reduction of nitrite with iodide to NO and subsequent measurement of the increase in NO concentration with the electrode. As can be seen from the data in Table 1, the results obtained using these two techniques are in close agreement.

Although the characteristic fingerprint (due to vibrational coupling) absorbance spectrum observed for acidified NO_2^- solutions is well-known and has been assigned to the formation of HONO[19−21] it has in some cases been misinterpreted as the spectrum of NO, formed via disproportionation of NO_2^- in acidic medium.[1] To confirm that the observed fingerprint spectrum is indeed due to the presence of HONO rather than NO, the UV−vis spectra for deoxygenated 0.01 M $HClO_4$ saturated with NO (a) or containing 2×10^{-3} M nitrite (b) were compared. As shown in Figure 1, the intensity of the fingerprint spectrum is similar in both solutions, despite the large difference in NO concentration as measured with the NO electrode, viz., 1.5×10^{-3} and 2.7×10^{-6} M for (a) and (b), respectively. The extinction coefficient determined for a 2 mM NO_2^- solution at pH 2 (b) at 371 nm is 51 M^{-1} cm^{-1}, which is in good agreement with data reported in the literature.[20,21] We typically found that a 1.5 mM solution of NO (prepared by bubbling NO gas over a longer period of time through weakly acidic aqueous solution at room temperature) could easily contain up to 2 mM nitrite, even though all solutions were prepared under argon or nitrogen atmosphere. In particular, we were unable to lower the NO_2^- concentration below 0.3 mM, even though all precautions available in our laboratory were employed to exclude oxygen from the system (i.e., including ultrasonic treatment, freeze−pump−thaw cycles, and a solid KOH covered catalyst to remove higher nitric oxides from the NO gas stream). Thus the fingerprint spectrum observed in case (a) can only be assigned to the presence of nitrite as impurity.

The reaction of $B_{12}-H_2O$ with solutions of NO was studied spectrophotometrically, and the kinetics of the reaction was followed using conventional stopped-flow techniques, as in our earlier studies.[22,23] As can be seen from Figure 2, the product spectra obtained for the reactions of $B_{12}-H_2O$ with NO-saturated solution (containing nitrite impurities) or with solutions containing only nitrite are indeed identical. Figure 3 shows typical kinetic traces recorded for the reaction with NO (containing nitrite impurities) and only nitrite. Figure 3a demonstrates that the reaction with NO-saturated solution, containing a low concentration of NO_2^-, does not give good first-order kinetics, although an appropriate excess of NO was used in order to establish pseudo-first-order conditions. Under identical experimental conditions, but using only nitrite at a higher concentration instead of an NO-saturated solution, good first-order behavior was found (Figure 3b). As can be seen from the kinetic data summarized in Table 2, saturation of the latter nitrite solution with NO at three different pH values resulted in no increase in the observed rate constant, after an appropriate correction for the increase in nitrite concentration (introduced along with NO) was made.

It has been reported in the literature[24] that reactions of various cobalt complexes with nitric oxide carried out in the presence of excess NO can lead to the formation of an NO-coordinated intermediate, followed by a rapid conversion into a nitrite derivative. Such processes may be too fast to be observed on a stopped-flow time scale under the conditions described above. For this reason we repeated our kinetic measurements using an excess of vitamin B_{12a} in comparison to NO. However, we did not find any evidence for the formation of any intermediates, even at lower temperature (10 °C) and the shortest experimentally accessible reaction times (down to 20 ms). As in experiments with NO (or NO_2^-) in excess, the rate constants obtained for reaction with the diluted NO-saturated solutions containing nitrite impurities and solutions containing only nitrite (at the appropriate concentration) were very similar, and the B_{12a}:NO_2^- molar ratio (rather than the B_{12a}:NO molar ratio) was a critical parameter for the occurrence of pseudo-first-order kinetics.

Table 3 summarizes the pseudo-first-order rate constants obtained for the reaction of $B_{12}-H_2O$ (or $B_{12}-OH$, respectively) with NO_2^- under different experimental conditions. The second-order rate constants calculated from these data are in close agreement with literature data[25] (some variation observed in the literature data can be accounted for in terms of different experimental conditions, i.e., different pH, ionic strength, and nature of the salt used to maintain the ionic strength). We conclude that, under the conditions used in this study, vitamin $B_{12}-H_2O$ reacts not with NO but only with NO_2^- impurities present in NO solutions.

Preliminary experiments on reduced vitamin B_{12r} (in which the cobalt atom is in the 2+ oxidation state) indicated significant differences both in the UV−vis spectral changes and in the kinetics of the reaction with NO_2^- and NO, respectively. The reaction with nitrite is slow, and the final UV−vis spectrum resembles that of a mixture of $B_{12}-NO_2^-$ and $B_{12r}-NO$, i.e., a mixture of Co(III) and Co(II), respectively. In contrast, the reaction between vitamin B_{12r} and NO occurs much more rapidly. When the reaction was carried out under oxygen-free conditions, a rapid formation of $B_{12r}-NO$ with a characteristic UV−vis spectrum was observed. When air was allowed into the system, the products formed in both reactions (i.e., with NO_2^- and NO under anaerobic conditions) were oxidized to give a final spectrum identical with that of $B_{12}-NO_2^-$. It follows from these observations that the reduced form of vitamin B_{12a} does apparently bind NO.[1−3] However, our finding that aquacobalamin (vitamin B_{12a}) does not bind NO may have important consequences for the studies referred to in the introductory comments,[1−8] in which a direct interaction between vitamin B_{12a} and NO was assumed.

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Supporting Information Available: Figures 2 and 3 showing spectral changes and kinetic traces for the reaction of vitamin B_{12a}, respectively, and Tables 2 and 3 listing the rate constants for the reactions of vitamin B_{12a}. This material is available free of charge via the Internet at http://pubs.acs.org.

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