Novel Reactions of Nitric Oxide in Biological Systems

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The reaction between nitric oxide and the naturally occurring carotenoid β-carotene is shown by EPR and optical spectroscopy to produce a series of nitroxides with concomitant loss in conjugation of the β-carotene molecule, with a rate constant of *ca.* 10^3 dm^3 mol^{-1}.

Nitric oxide is a free radical which has an important function in a wide range of physiological and pathophysiological processes. These include regulation of vascular tone, a process mediated by the reaction of NO with the haem iron of the enzyme guanylate cyclase.[1] Some of the cytotoxic effects of NO have been proposed to be mediated by its reaction with superoxide to produce peroxynitrite, a powerful oxidant[2] and nitrating agent.[3] Peroxynitrite has been implicated in the oxidation of LDL (low density lipoprotein),[4] which leads to the formation of atherogenic plaques. We report here another type of reaction between NO and conjugated diene systems which may be of physiological relevance.

We have recently shown that nitroxyl radicals [R_2NO] are readily formed from NO and 2,5-dimethyl-2,4-hexadiene, and that despite a suggestion that this reaction requires the presence of NO_2,[5] we know that it proceeds readily in the complete absence of NO_2.[6] These reactions are related to those of the 'cheletropic traps' developed by Korth *et al.*,[7] but specifically do not involve aromatic compounds. In order to form 1:1 cyclic derivatives we need either polyenes that can exist in *cis*-configurations or those that are constrained into a *cis*-form. We have selected three important compounds that are widely distributed in nature, namely, vitamin A, vitamin D_3 and β-carotene. These all react with trace quantities of NO to give strong EPR signals characteristic of nitroxyl radicals.

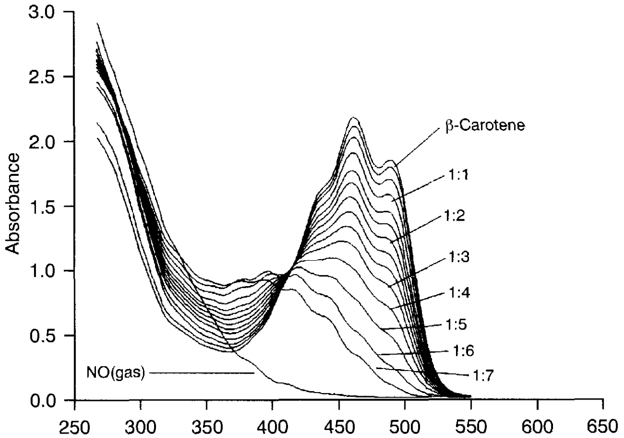

Fig. 1 Bleaching of β-carotene (30 μmol dm^{-3}) by NO. β-Carotene in tetrachloromethane was rendered anaerobic by alternating cycles of evacuation and nitrogen purging. The solution was transferred anaerobically to a degassed cuvette sealed with a rubber vaccine cap, such that the cuvette was completely filled leaving no gas phase. Small volume additions of a saturated NO solution in tetrachloromethane (the NO concentration was calculated to be 15.5 mmol dm^{-3} using the respective Ostwald coefficient of 0.345.[9] This was confirmed with a titration of ferrous myoglobin with the NO solution.) were added to the β-carotene solution to give the molar ratios indicated (β-carotene : NO). The spectra were recorded on a Varian Cary 5E UV–VIS–NIR spectrophotometer. For the final spectrum NO gas was bubbled through the solution.

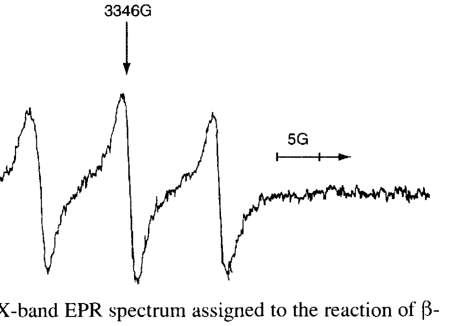

Fig. 2 First derivative X-band EPR spectrum assigned to the reaction of β-carotene with pure nitric oxide in tetrachloromethane at room temp.

Here, we concentrate on the reactions with β-carotene. As expected from the above, each molecule can react with a number of NO molecules (probably up to seven), as shown by optical studies (Fig. 1), the decrease in absorbance at 460 and 490 nm, and the concomitant increase in absorbance below 400 nm, suggests that a transition from the highly conjugated system of β-carotene (11 conjugated double bonds) to a lesser conjugated system occurs. What appears to be an isosbestic point is seen at 423 nm. However, this disappears at molar ratios above 1:4 (Fig. 1), thus suggesting the formation of a number of spectrally distinct species. The products of the reaction were stable for relatively long periods of time (> 1 h), and also after exposure to air.

In the initial stages of 'bleaching' (< 1:1) there is a concomitant growth in the EPR signal, which comprises three broad (^{14}N) features with shoulders suggesting several different species (Fig. 2). The initial EPR spectra are isotropic, but all show a clear broadening of the outer lines indicating some restricted motion. Further addition gives line broadening and some fluctuation in signal intensity, as expected if several R_2NO units are formed within each molecule. This reaction was not a result of residual NO_2 in the system. We suggest that a series of nitroxyls are arbitrarily formed initially, probably having 5,7–23 membered rings depending on the particular molecular configuration encountered by NO. This explains the immediate onset of extensive loss of conjugation indicated by the optical changes.

Our present concern is not to establish the precise structures of these derivatives, but simply to show that the initial reaction between β-carotene and NO gives rise to stable nitroxyl radicals. Stopped flow experiments in which β-carotene in tetrachloromethane was rapidly mixed with NO in the same solvent showed a bleaching of the absorbance at 460 nm. This kinetic process is complex and is NO concentration dependent, yielding an apparent second-order rate constant of approximately 5×10^3 dm^3 mol^{-1} s^{-1}.

There are a number of possible implications from our findings. Certainly there seems to be a real chance of such reactions occurring *in vivo*. For example β-carotene is a constituent of the protein lipid complex, LDL the *in vivo* cholesterol transport particle. Moreover, conjugated dienes, of which β-carotene is only one, are widespread particularly under conditions of oxidative stress. Under such conditions membrane and LDL peroxidation is accompanied by conjugated diene formation in the fatty acyl chains.[8] Nitric oxide is more soluble in the hydrophobic phase presented by lipids and thus conjugated diene–NO reactions would be favoured.

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