Fast Formation of NO in Reactions of Alkyl Nitrites with Ascorbic Acid and Analogues

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Reaction of alkyl nitrites and other NO-carriers with conjugation-stabilized enediols such as ascorbic acid at neutral or basic pH gives rise to fast production of NO.

Only until recently, such scant attention as was paid to the poisonous gas nitric oxide was largely in relation to environmental pollution issues. Since then, its identification as the blood pressure regulator EDRF (endothelium-derived relaxing factor)[1,2] and the discovery of its roles in neurotransmission, digestion, the immune response and the regulation of platelet aggregation,[3] have led to NO being the object of intense multidisciplinary research.[4]

It has been known for many years that organic nitrate and nitrite esters cause muscle relaxation by releasing NO.[5] Some of these compounds are also known to produce NO when reacted with biological reductones (*i.e.* conjugation stabilized enediols) in acidic media.[6] Alkyl nitrites, on the other hand, typically lose their NO group by transferring it to amines, carbanions, thiols and other species, rather than by releasing molecular NO through homolytic cleavage of the O–NO bond.[7] It has been assumed that this latter class of vasodilators may act by reaction with thiols to afford unstable nitrosothiols, and that it is this latter compounds that release the vasodilatory NO.[1,8] In the work here described we found *in vitro* evidence of other paths by which alkyl nitrites may generate NO rapidly and in quantitative yield in basic media, namely by reaction with biological reductones such as ascorbic acid and catechol.

In the course of a study of the reactivities of alkyl nitrites and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (MNTS) with phenols and related species in neutral and basic media, we found that the former compounds reacted faster with catechol and ascorbic acid than expected for phenols of similar pK_a. The reaction between ascorbic acid and 2-ethoxyethyl nitrite (EEN) was studied by conventional spectrophotometry recording the decrease in absorbance at 270 nm due to the ascorbic acid disappearance. Experiments were performed under pseudo-first-order conditions with [EEN] ≫ [ascorbic acid]. At pH 10–12 and 25 °C, the reaction, which is of first order with respect to both reagents (Fig. 1), has a bimolecular rate constant of 416 $dm^3\ mol^{-1}\ s^{-1}$. The reactive ascorbic acid species in this reaction is the dianion (Scheme 1), since the observed influence of pH on the pseudo-first-order rate constant k_o (Fig. 2) implies a pK_a of 12.0, in good agreement with previously published values for the second deprotonation of ascorbic acid.

Determination of NO_2^- in the final reaction mixtures [2 × 10^{-3} mol dm^{-3} EEN and 1 × 10^{-3} mol dm^{-3} ascorbic acid at pH 10.95 (1 × 10^{-3} mol dm^{-3} carbonate–bicarbonate buffer)] by Shinn's method[9] showed yields of about 70%, which increased to about 85% if the final reaction mixture was bubbled with oxygen for a few minutes, but was reduced to less than 5% if Ar had been bubbled through the reaction. We inferred that one of the products of the reaction was a gas that is transformed to NO_2^- in the presence of oxygen but is removed from the reaction mixture by bubbling with inert gas. This gas was identified as NO by bubbling Ar through the reaction mixture and, with O_2 added, into a solution of ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] of pH 7, which produced a green solution showing reaction with NO.[10] Analysis by Shinn's method showed the reaction mixture to be left with no NO in this experiment, but the ABTS test is not quantitative because most NO bubbles through the ABTS solution without reacting; NO-selective electrode measurements of a reaction at pH 12 with [ascorbic acid] = 0.1 mol dm^{-3} and [EEN] = 9 × 10^{-6} mol dm^{-3} showed an NO yield of at least 60%. From these yields it is possible to conclude that two molecules of EEN are needed per molecule of ascorbate. A possible mechanism for this reaction involves initial nitrosation of the dianion followed by homolytic cleavage of the O–NO bond to give NO and a radical anion which would be further oxidised to dehydroascorbic acid by another molecule of EEN. Dehydroascorbic acid could not be identified due to its high instability.

Similar behaviour was observed for the reaction between EEN (2 × 10^{-3} mol dm^{-3}) and catechol (1 × 10^{-3} mol dm^{-3}) at pH 13: final reaction mixtures contained nitrite yields of about 80% if not bubbled with Ar, Ar bubbling reduced this figure to <4%, and the ABTS test confirmed NO formation.

The reaction between catechol and MNTS (which in nitrosation reactions transfers the NO group at a rate similar to EEN) was studied kinetically. Because the high absorbance of the reaction products prevents spectrophotometric study, the reaction was followed by HPLC using a Beckman System Gold apparatus with a Beckman Reverse Phase 5 μm Ultrasphere C18 column and a UV–VIS detector recording the fall in MNTS concentration at 250 nm. Reaction mixtures of known pH

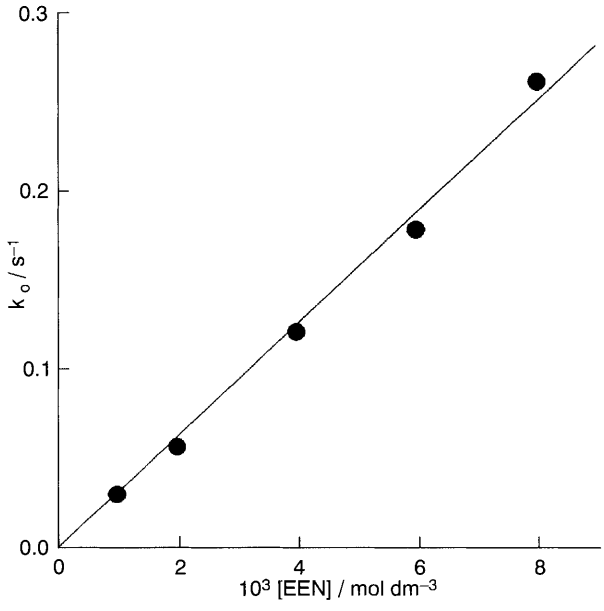
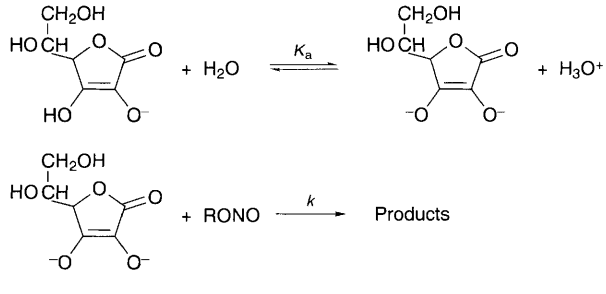


Fig. 1 Influence of EEN concentration on the pseudo first order rate constant k_o of its reaction with ascorbic acid (5 × 10^{-5} mol dm^{-3}) at pH 10.95 and 25 °C. Medium: 10^{-2} mol dm^{-3} carbonate/bicarbonate buffer with 10% acetonitrile.



Scheme 1

between 9 and 10.5 containing 5×10^{-4} mol dm^{-3} MNTS and catechol concentrations in the range (5–20) $\times$ 10^{-3} mol dm^{-3} were analysed at various reaction times using a 2 cm^3 min^{-1} flow of 50:50 (*v/v*) methanol–water as mobile phase. The observed kinetics were similar to those of the reaction between EEN and ascorbate, with bimolecular rate constants of 0.6 ± 0.08 dm^3 mol^{-1} s^{-1} for reaction with the monoanion and 410 ± 40 dm^3 mol^{-1} s^{-1} for reaction with the dianion. Reaction involves the MNTS –NO group (*i.e.* nitrosation of catechol), since analysis of the final reaction mixtures by HPLC showed quantitative formation of *N*-methyl-*p*-toluenesulfonamide. The *o*-quinone derived from catechol, which is expected to be formed in this reaction, is known to be very unstable and therefore was not identified.

Both ascorbic acid and catechol are biological reductones, *i.e.* conjugation-stabilized enediols, present in mammals and other animals.[6] The above reactions may therefore constitute an alternative to the nitrosothiol pathway for the *in vivo* formation of NO from alkyl nitrites (and possibly from other NO-bearing vasodilators also). Note that a considerable range of reaction rates could be achieved by varying the NO-donor: the rate constants reported above for EEN and MNTS imply that at similar concentrations complete reaction would take just a few seconds at lower pHs using alkyl nitrites with electron-withdrawing groups in the β-position, such as Cl_2CHCH_2ONO, which effect *C*-nitrosation of diketones about 1000 times faster than EEN.[11]

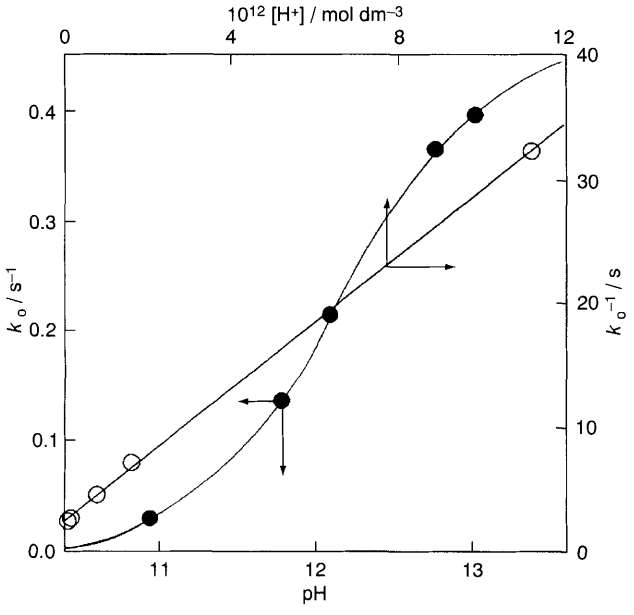


Fig. 2 Influence of acidity on the pseudo first order rate constant of the reaction of EEN (10^{-3} mol dm^{-3}) with ascorbic acid (5×10^{-5} mol dm^{-3}) at 25 °C in the presence of 10% acetonitrile. (●) k_o plotted against pH; (○) $1/k_o$ plotted against [H$^+$].

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