Complexes of •NO with Nucleophiles as Agents for the Controlled Biological Release of Nitric Oxide. Vasorelaxant Effects

Chris M. Maragos,† Deborah Morley,‡ David A. Wink,† Tambra M. Dunams,† Joseph E. Saavedra,† Aaron Hoffman,§ Alfred A. Bove,‡ Lawrence Isaac,∥ Joseph A. Hrabie,⊥ and Larry K. Keefer*,†

Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702, Cardiology Section, Department of Medicine, Temple University, Philadelphia, Pennsylvania 19140, National Heart, Lung and Blood Institute, Bethesda, Maryland 20892, Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612, and PRI/DynCorp, NCI-FCRDC, Frederick, Maryland 21702. *Received April 3, 1991*

Selected nucleophile/nitric oxide adducts [compounds which contain the anionic moiety, $XN(O^-)N{=}O$] were studied for their ability to release nitric oxide spontaneously in aqueous solution and for possible vasoactivity. The diversity of structures chosen included those in which the nucleophile residue, X, was that of a secondary amine [Et_2N, as in $[Et_2NN(N{=}O)O]Na$, **1**], a primary amine [^{i}PrHN, as in $[^i PrHNN(N{=}O)O]Na$, **2**], a polyamine, spermine [as in the zwitterion $H_2N(CH_2)_3NH_2^+(CH_2)_4N[N(N{=}O)O^-](CH_2)_3NH_2$, **3**], oxide [as in $Na[ON(N{=}O)O]Na$, **4**], and sulfite [as in $NH_4[O_3SN(N{=}O)O]NH_4$, **5**]. The rate constants (k) for decomposition in pH 7.4 phosphate buffer at 37 °C, as measured by following loss of chromophore at 230–260 nm, were as follows: **1**, 5.4×10^{-3} s^{-1}; **2**, 5.1×10^{-3} s^{-1}; **3**, 0.30×10^{-3} s^{-1}; **4**, 5.0×10^{-3} s^{-1}; and **5**, 1.7×10^{-3} s^{-1}. The corresponding extents of nitric oxide release (E_{NO}) were 1.5, 0.73, 1.9, 0.54, and 0.001 mol/mol of starting material consumed, respectively, as determined from the integrated chemiluminescence response. Vasodilatory activities expressed as the concentrations required to induce 50% relaxation in norepinephrine-constricted aortic rings bathed in pH 7.4 buffer at 37 °C (EC_{50}) were as follows: **1**, 0.19 μM; **2**, 0.45 μM; **3**, 6.2 μM; **4**, 0.59 μM; and **5**, 62 μM. Vasorelaxant potency (expressed as $1/EC_{50}$) was strongly correlated with the quantity of •NO calculated from the physicochemical data to be released in the interval required to achieve maximum relaxation at the EC_{50} doses ($r = 0.995$). This suggests that such nucleophile/•NO adducts might generally be useful as vehicles for the nonenzymatic generation of nitric oxide, in predictable amounts and at predictable rates, for biological purposes. The particular significance for possible drug design is underscored in the very favorable potency comparison between several of these agents and the established nitrovasodilators sodium nitroprusside and glyceryl trinitrate (EC_{50} values of 2.0 and >10 μM, respectively) in parallel aortic ring tests.

Introduction

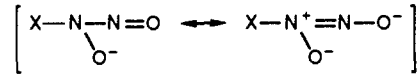
Nitric oxide (•NO) has recently been implicated in a variety of important bioregulatory processes, including normal physiological control of blood pressure, macrophage-induced cytostasis and cytotoxicity, and neurotransmission.[1] We have been interested in the possibility that agents of structure $XN(O^-)N{=}O$, where X is a nucleophile residue, might serve as vehicles for the controlled delivery of •NO into a biological system and thus display useful pharmacological effects. Syntheses of several such compounds by reaction of nitric oxide with nucleophiles have been described.[2] The products have in some cases been shown to be capable of regenerating •NO upon dissolution in simple aqueous buffers.[2,3] We have postulated, therefore, that such agents might serve as promising biomedical research tools and potential drugs the action of which should be predictable from the quantities of •NO generated during exposure.

We have now measured the rates and extents of spontaneous nitric oxide release from five such compounds at pH 7.4 and 37 °C and studied their effects on the isolated rabbit aorta. In the present report, we describe the potent vasorelaxant action of certain members of the series and illustrate the successful prediction of their biological properties from data on the kinetics and stoichiometry of their decomposition.

Results

The compounds chosen for these investigations are depicted in Scheme I. They include five representative structures in which a nitric oxide dimer (O=NN=O) is formally bound to a nucleophile residue (X^-) via a nitrogen atom (a secondary or primary amine or a polyamine), an oxygen (oxide), or sulfur (sulfite). All were synthesized according to literature methods except the polyamine adduct **3**, which has not previously been described; this zwitterionic material was prepared by exposing a tetra-

Scheme I. Nucleophile/•NO Adducts of Structure $XN(O^-)N{=}O$ Investigated Here for Vasorelaxant Effects



compound	X
1	Et_2N[a]
2	^{i}PrHN[a]
3	$H_2N(CH_2)_3NH_2^+(CH_2)_4$ N $H_2N(CH_2)_3$
4	^-O[b]
5	^-O_3S[c]

[a] Monosodium salt. [b] Disodium salt. [c] Bis(ammonium) salt.

† National Cancer Institute.
‡ Temple University.
§ National Heart, Lung and Blood Institute.
∥ University of Illinois.
⊥ PRI/DynCorp.

(1) (a) *Nitric Oxide from* L-*Arginine: A Bioregulatory System*; Moncada, S., Higgs, E. A., Eds.; Excerpta Medica, International Congress Series 897; Elsevier Science Publishers B. V.: Amsterdam, 1990. (b) Marletta, M. A.; Tayeh, M. A.; Hevel, J. M. Unraveling the Biological Significance of Nitric Oxide. *Biofactors* **1990**, *2*, 219–225. (c) Ignarro, L. J. Nitric Oxide. A Novel Signal Transduction Mechanism for Transcellular Communication. *Hypertension (Dallas)* **1990**, *16*, 477–483.

(2) Drago, R. S. Reactions of Nitrogen(II) Oxide. In *Free Radicals in Inorganic Chemistry*; Advances in Chemistry Series, Number 36; American Chemical Society: Washington, DC, 1962; pp 143–149.

(3) Hansen, T. J.; Croisy, A. F.; Keefer, L. K. N-Nitrosation of Secondary Amines by Nitric Oxide via the 'Drago Complex'. In *N-Nitroso Compounds: Occurrence and Biological Effects*; Bartsch, H., O'Neill, I. K., Castegnaro, M., Okada, M., Eds.; IARC Scientific Publications No. 41; International Agency for Research on Cancer: Lyon, 1982; pp 21–29.

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Table I. Observed Vasorelaxant Properties and Physicochemical Data for Various Nucleophile/•NO Complexes at 37 °C in pH 7.4 Buffer[a]

compd	λ_{max}, nm	ϵ_{max}, mM^{-1} cm^{-1}	k, s^{-1}	$t_{1/2}$, min	E_{NO}	log EC_{50}, M	EC_{50}, μM	R_M, %	t, min
1	250	6.5	$(5.4 \pm 0.2) \times 10^{-3}$	2.1	1.5 ± 0.11	-6.72 ± 0.17	0.19	86 ± 5	3.1
2	252	8.7	$(5.1 \pm 0.4) \times 10^{-3}$	2.3	0.73 ± 0.04	-6.35 ± 0.26	0.45	85 ± 9	3.6
3	252	8.5	$(0.30 \pm 0.02) \times 10^{-3}$	39	$(1.90 \pm 0.00_3)$[b]	-5.21 ± 0.22	6.2	56 ± 9	3.3
4	237	6.1	$(5.0 \pm 0.2) \times 10^{-3}$	2.3	0.54 ± 0.04	-6.23 ± 0.28	0.59	74 ± 10	3.9
5	259	7.1	$(1.7 \pm 0.2) \times 10^{-3}$	6.8	$0.001 \pm 0.000_3$	-4.21 ± 0.26	62	58 ± 13	2.9

[a] k is the observed rate constant for loss of chromophore, $t_{1/2}$ is the half-life, E_{NO} is the number of moles of nitric oxide generated in the decomposition per mole of starting material consumed (N = 8 or 9), EC_{50} is the concentration required to produce 50% relaxation in a norepinephrine-constricted strip of isolated rabbit aorta (N = 5 or 6), R_M is the peak relaxation observed (i.e. that at the maximal concentration employed—10^{-5} M for 1–4 and 10^{-4} M for 5), and t is the average elapsed time between dosage and achievement of maximum relaxation at concentrations near the EC_{50}. Error limits are SD's for the k's and SE's for the log EC_{50} and R_M values. [b] Decomposition was too slow at pH 7.4 to integrate the chemiluminescence response over infinite time, so the value given was determined at pH 2.

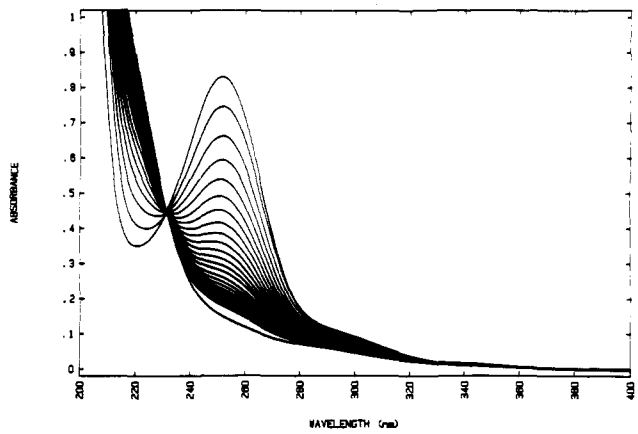

Figure 1. Ultraviolet spectra of **3** at intervals of 5 min (and at infinite time) after dissolution in pH 7.4 phosphate buffer at 37 °C.

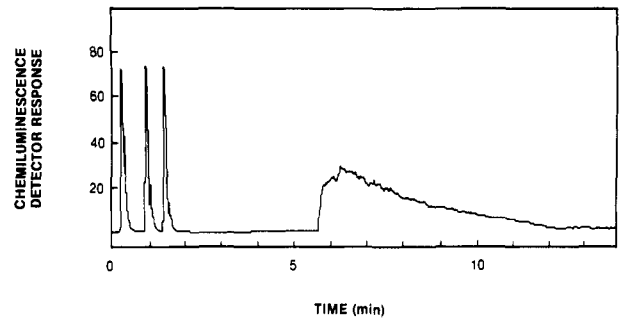

Figure 2. Chemiluminescence detector response to nitric oxide produced as a function of time after dissolution of **4** at 10 μM in 1 mL of pH 7.4 phosphate buffer at 37 °C. The three peaks at 0–2 min are replicates of the 0.361 nmol nitric oxide quantitative reference standard. Compound **4** was added at 5.5 min.

hydrofuran solution of spermine to 5 atm of nitric oxide at room temperature in a modification of the procedure developed by Drago and co-workers.[4-6]

Rates and Extents of Nitric Oxide Release from Nucleophile/•NO Complexes. While all five compounds are stable as solids, decomposition occurs when they are dissolved. Rates of starting material disappearance in pH 7.4 phosphate buffer at 37 °C were measured by following the loss of the intense chromophore these compounds characteristically show at λ_{max} 230–260 nm (ϵ_{max} 6000–9000 M^{-1} cm^{-1}). A typical example of the spectral changes occurring with time is shown in Figure 1. First-order behavior was observed for all five compounds, the correlation coefficient for the ln (absorbance − absorbance$_\infty$) versus time plots being consistently ≥0.998. The observed rate constants (k) and half-lives ($t_{1/2}$) are summarized in Table I.

Rate constants were found to vary, sometimes dramatically, with pH. While a complete description of the complicated dependence on hydrogen ion activity is beyond the scope of this report, one consistent generalization did emerge: rate constants invariably increased as the pH was lowered. This fact was used to our advantage in the present studies. Compounds found to decompose very rapidly at pH 7.4 could be stabilized during the initial dissolution and dilution phases of the kinetic and biological studies by keeping the medium alkaline and then mixing rapidly with excess pH 7.4 buffer to start the reaction. Using this approach, reproducible absorbance values at time = 0 were obtained for all five test substances.

Extents of nitric oxide release under the same conditions were also determined. A thermostated (37 °C) reactor was purged with helium to remove traces of oxygen and nitric oxide and then charged with the nitric oxide complex to be studied. Deaerated phosphate buffer (pH 7.4) at 37 °C was then introduced via a septum to start the reaction. The nitric oxide evolved was quantified by sweeping purged gases continuously into a chemiluminescence detector. Figure 2 is an example of the recorder trace observed. Summation of the area under these curves from time zero to infinity provided data on the number of moles of nitric oxide generated (E_{NO}) per mole of starting material employed. These values are given in Table I. It should be noted that **3** decomposed too slowly to permit accurate integration of its chemiluminescence trace at pH 7.4, so its E_{NO} was determined at pH 2.0 instead. This may be important because lowering the pH has been found to enhance apparent •NO recovery for the other compounds. For example, the E_{NO} for compound **1** at pH 7.4 was 1.5, whereas at pH 2.0 it increased to 2.1. Hence the Table I measurement of E_{NO} = 1.9 for **3** may overestimate its value at pH 7.4.

Control experiments with nitrous oxide (N_2O) and freshly purified nitrogen dioxide (NO_2) showed that under the conditions used here these potential products of **1–5** decomposition gave molar response factors in the chemiluminescence detector of only 10^{-5}% and 1% that of nitric oxide, respectively, and thus that they would not significantly interfere with the •NO determinations.

Vasoactivity of Nucleophile/Nitric Oxide Complexes. To test for the predicted vasorelaxant effect of these compounds, a standard isolated vascular ring preparation was employed.[7] Segments of rabbit thoracic aorta

(4) Drago, R. S.; Paulik, F. E. The Reaction of Nitrogen(II) Oxide with Diethylamine. *J. Am. Chem. Soc.* **1960**, *82*, 96–98.
(5) Drago, R. S.; Karstetter, B. R. The Reaction of Nitrogen(II) Oxide with Various Primary and Secondary Amines. *J. Am. Chem. Soc.* **1961**, *83*, 1819–1822.
(6) Longhi, R.; Ragsdale, R. O.; Drago, R. S. Reactions of Nitrogen(II) Oxide with Miscellaneous Lewis Bases. *Inorg. Chem.* **1962**, *1*, 768–770.

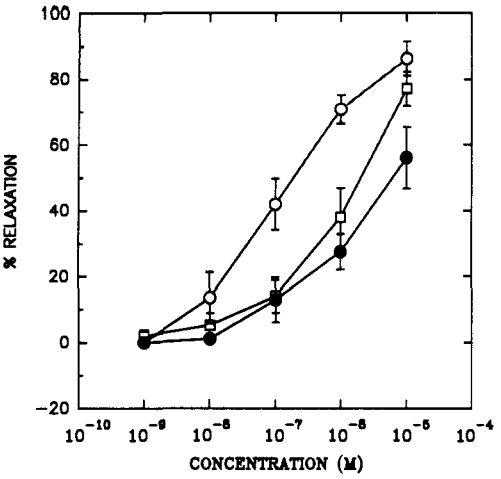

Figure 3. Vasorelaxant activities of compound 1 (○), compound 3 (●), and sodium nitroprusside (□) as a function of concentration in the standard rabbit aortic ring test. (Remaining dose–response curves are available as supplementary material.) Values for percent relaxation are given as means ± SE for four to six replicates.

were placed in a pH 7.4 modified Krebs buffer at 37 °C and constricted with norepinephrine against a 10-g preload. Dose–response curves were obtained for sodium nitroprusside and glyceryl trinitrate, two established vasodilators,[8] as well as for 1–5 by placing the compounds into the baths and measuring the extent of relaxation over the range 10^{-9}–10^{-5} M (10^{-8}–10^{-4} M for 5). Control experiments with $NaNO_2$ indicated that the oxidized end product of nitric oxide release under these conditions, the nitrite ion, did not have a significant vasodilator effect at the concentrations employed.

Vasorelaxant activities of compounds 1 and 3 are compared with those of the standard nitrovasodilator sodium nitroprusside in Figure 3. Similar plots from parallel tests with 2, 4, 5, •NO, and glyceryl trinitrate are available as supplementary material. Potencies expressed as the concentrations required to induce 50% relaxation in the rings (EC_{50} values) are summarized in Table I, as are the times (t) required for the EC_{50} doses to achieve their maximum effect and the peak relaxations registered at the highest concentrations tested (R_M).

Discussion

Certain nucleophile/•NO complexes have been reported to decompose with generation of nitric oxide under appropriate conditions.[2,3] In theory, as many as 2 mol of •NO can be liberated per mole of complex, as depicted in eq 1. We have explored the degree to which the vascular

$$XN(O^-)N{=}O \rightarrow X^- + 2(\cdot NO) \qquad (1)$$

action of selected nucleophile/•NO complexes could be predicted from their physicochemical properties. The compounds examined (Scheme I) cover a broad range of rates and extents of nitric oxide release, with half-lives at pH 7.4 and 37 °C from 2.1 to 39 min and E_{NO} values from 0.001 to near the theoretical maximum of 2.0 (Table I).

Since nitric oxide has been identified with the action of the endothelium-derived relaxing factor (EDRF),[1] one might expect the vasodilatory activity of these compounds to correlate with the total amount of nitric oxide released spontaneously under similar conditions. In general, the quantity of nitric oxide generated (Q) during time t (the time required to achieve maximal relaxation at concentrations near the EC_{50}) can be calculated from the data of Table I via eq 2, where the quantity $(1 - e^{-kt})$ represents

$$Q = E_{NO}C_0V(1 - e^{-kt}) \qquad (2)$$

the fraction of the administered dose that decomposes during time t, E_{NO} is the number of moles of •NO produced per mole of complex consumed, V is the volume of the solution, and C_0 is the initial concentration of the test agent.[9]

To compare potencies among different •NO-releasing compounds, it is convenient to define a *relative* nitric oxide release parameter, F, which is equal to the fraction, Q/C_0V. This is done by rearranging eq 2, as in eq 3. As we shall

$$F = Q/C_0V = E_{NO}(1 - e^{-kt}) \qquad (3)$$

now show, this parameter correlates remarkably well with in vitro vasoactivity for complexes 1–5.

As a starting point for our study, we chose 1. Its k value indicated that 63% should decompose during time t, and its measured E_{NO} was 1.53 mol of •NO per mole of compound consumed. Therefore, its F value was calculated to be 0.96 molecules of nitric oxide produced during time t for each molecule of 1 added at time zero. This was the largest F value calculated for the five nucleophile/•NO complexes studied. As expected, 1 was associated with the greatest vasoactivity (lowest EC_{50}) in the aortic ring test.

Both the reactivity and the vasorelaxant potency could be altered substantially by changing the identity of the nucleophile (the X residue of Scheme I). For example, the k and E_{NO} values for the primary amine derivative 2 indicated that about half as much nitric oxide should be generated during the first 3 min after dissolution as was observed for 1. Thus the EC_{50} for 2 should be twice that of 1, a result observed empirically (Table I). This structure–activity variation is also important from the viewpoint of byproduct pharmacology. A disadvantage of anion 1 for some applications is that its decomposition products in air include the carcinogen, *N*-nitrosodiethylamine.[10] Carcinogenic nitrosamines are not among the anticipated or observed decomposition products of 2, however,[10] making it a potentially more attractive research tool.

Another structural variant tested was the slow-release agent 3. In this case, nitric oxide is complexed with spermine. Thus the decomposition of 3 in solution (eq 1) should produce not only •NO but also free spermine,[11] a

(7) Tallarida, R. J.; Sevy, R. W.; Harakal, C.; Bendrick, J.; Faust, R. The Effect of Preload on the Dissociation Constant of Norepinephrine in Isolated Strips of Rabbit Thoracic Aorta. *Arch. Int. Pharmacodyn. Ther.* **1974**, *210*, 67–74.

(8) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th ed.; Gilman, A. G., Rall, T. W., Nies, A. S., Taylor, P., Eds.; Pergamon Press: New York, 1990.

(9) The exponential form results from the observed first-order character of the decomposition reactions. A full derivation of eqs 2–4 and their application is deposited as supplementary material.

(10) Ragsdale, R. O.; Karstetter, B. R.; Drago, R. S. Decomposition of the Adducts of Diethylamine and Isopropylamine with Nitrogen(II) Oxide. *Inorg. Chem.* **1965**, *4*, 420–422.

(11) While the polycationic starting material does contain secondary amino groups and in fact can be converted to a nitrosamine in a pathway analogous to that for 1, nitrosating agents to which it is exposed appear to be largely consumed in the deamination of primary amino groups, with stable *N*-nitroso derivatives being rather minor products. Hildrum, K. I.; Scanlan, R. A.; Libbey, L. M. Identification of γ-Butenyl-(β-propenyl)nitrosamine, the Principal Volatile Nitrosamine Formed in the Nitrosation of Spermidine or Spermine. *J. Agric. Food Chem.* **1975**, *23*, 34–37.

nucleophilic natural product with a number of important bioeffector roles in its own right,[12] including hypotensive activity.[13] Because its rate of nitric oxide generation was 1 order of magnitude smaller than that of 1, the relative extent of •NO release during the time course of the aortic ring experiments was greatly decreased. Thus complex 3 should be considerably less potent than 1, a conclusion supported by a comparison of the EC_{50}'s. Control experiments showed that free spermine was devoid of measurable activity under these conditions, indicating that the action of 3 was attributable to nitric oxide release.

Another compound included in the present investigation was "Angeli's salt", 4, the oxide adduct [$XN(O^-)N{=}O$, X = O^{2-}]. While decomposition of this complex according to eq 1 would be expected to yield •NO and H_2O, most literature reports indicate that $N_2O_3^{2-}$ disproportionates exclusively to NO^- (which dimerizes to N_2O) and NO_2^- at neutral pH.[14,15] However, Doyle and Mahapatro have presented evidence for dissociation of nitric oxide from $N_2O_3^{2-}$ when substrate concentration is low.[16] In support of the latter conclusion, •NO production as measured by chemiluminescence was extensive at micromolar concentrations (Figure 2). The F value calculated from the data of Table I indicates that 4 should be approximately one-third as active as 1. This correlates well with the 3-fold difference in EC_{50} (Table I). The results thus confirm the recent report of Vanin, et al., who have independently discovered the vasorelaxant properties of Angeli's salt.[17]

Compound 5, while it has the appropriate $XN(O^-)N{=}O$ structure and is known from the literature to decompose in aqueous solution, disproportionates to nitr*ous* oxide and sulf*ate* rather than regenerating the nitr*ic* oxide and sulf*ite* from which it is formed.[18-20] The reaction is so efficient that it has been recommended as an advantageous route to pure N_2O.[20] There is only isolated mention of any tendency to produce nitric oxide, and those reports describe harsh, nonphysiological conditions.[21] In the present experiments at pH 7.4 and 37 °C, 5 formed only minuscule amounts of nitric oxide (E_{NO} = 0.001 mol of •NO/mol of 5). From eq 3, very little •NO would be anticipated. Thus, 5 was predicted to be a very poor vasodilator, a result which was reflected in the large EC_{50} (Table I).

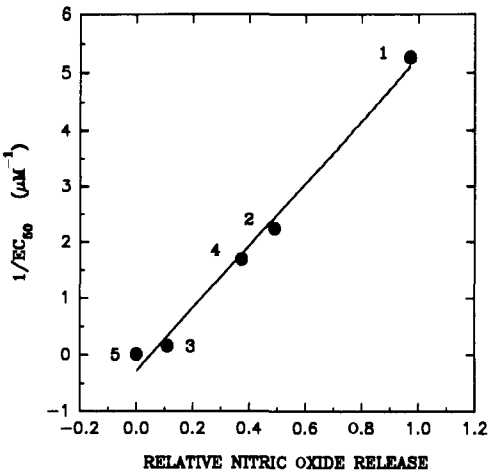

Figure 4. Correlation of vasodilatory activities ($1/EC_{50}$) for compounds 1–5 versus relative nitric oxide release (F) values calculated from eq 3. The correlation coefficient was 0.995.

The above pairwise comparisons suggest that potency may be linearly related to the quantity (Q) of nitric oxide released. If this is so, then the more •NO a given compound generates, the more potent its vasorelaxant action should be. As a corollary, equipotent concentrations of different compounds should generate equal quantities of nitric oxide. Taking the EC_{50}'s as specific equipotent initial concentrations (C_0) and rearranging eq 2, one can show that the relative •NO release factor, F, must be directly proportional to the reciprocal of the EC_{50}, as in eq 4. Thus

$$F = (Q/V)(1/EC_{50}) = E_{NO}(1 - e^{-kt}) \qquad (4)$$

a plot of F versus $1/EC_{50}$ should be a line of slope Q/V if potency is indeed directly proportional to the quantity of nitric oxide released. We have plotted these parameters for compounds 1–5 (Figure 4) and found excellent agreement between expectation and result (r = 0.995). Thus

(12) *The Physiology of Polyamines*; Bachrach, U., Heimer, Y. M., Eds.; CRC Press, Inc.: Boca Raton, FL, 1989; Vol. I and II.

(13) Marmo, E.; Berrino, L.; Cazzola, M.; Filippelli, A.; Cafaggi, G.; Persico, N.; Spadaro, R.; Nisticò, G. Cardiovascular and Respiratory Effects of Spermidine and Spermine: An Experimental Study. *Biomed. Biochim. Acta* **1984**, *43*, 509–515.

(14) Akhtar, M. J.; Bonner, F. T.; Hughes, M. N.; Humphreys, E. J.; Lu, C.-S. Kinetic and Isotopic Studies on the Reaction between Trioxodinitrate and the Hexaammineruthenium(III) Cation. *Inorg. Chem.* **1986**, *25*, 4635–4639.

(15) (a) Bonner, F. T.; Ravid, B. Thermal Decomposition of Oxyhyponitrite (Sodium Trioxodinitrate(II)) in Aqueous Solution. *Inorg. Chem.* **1975**, *14*, 558–563. (b) Hughes, M. N.; Wimbledon, P. E. The Chemistry of Trioxodinitrates. Part I. Decomposition of Sodium Trioxodinitrate (Angeli's Salt) in Aqueous Solution. *J. Chem. Soc., Dalton Trans.* **1976**, 703–707. (c) Hughes, M. N.; Wimbledon, P. E. The Chemistry of Trioxodinitrates. Part 2. The Effect of Added Nitrite on the Stability of Sodium Trioxodinitrate in Aqueous Solution. *J. Chem. Soc., Dalton Trans.* **1977**, 1650–1653. (d) Bazylinski, D. A.; Hollocher, T. C. Evidence from the Reaction between Trioxodinitrate(II) and ^{15}NO That Trioxodinitrate(II) Decomposes into Nitrosyl Hydride and Nitrite in Neutral Aqueous Solution. *Inorg. Chem.* **1985**, *24*, 4285–4288. (e) Bazylinski, D. A.; Goretski, J.; Hollocher, T. C. On the Reaction of Trioxodinitrate(II) with Hemoglobin and Myoglobin. *J. Am. Chem. Soc.* **1985**, *107*, 7986–7989. (f) Bonner, F. T.; Hughes, M. N. The Aqueous Solution Chemistry of Nitrogen in Low Positive Oxidation States. *Comments Inorg. Chem.* **1988**, *7*, 215–234.

(16) Doyle, M. P.; Mahapatro, S. N. Nitric Oxide Dissociation from Trioxodinitrate(II) in Aqueous Solution. *J. Am. Chem. Soc.* **1984**, *106*, 3678–3679.

(17) Vanin, A. F.; Vedernikov, Y. I.; Galagan, M. E.; Kubrina, L. N.; Kuzmanis, J.; Kalvins, I.; Mordvintsev, P. I. Angeli's Salt as a Nitrogen Oxide Producer in Animal Tissues (in Russian). *Biokhimiya (Moscow)* **1990**, *55*, 1408–1413.

(18) Ackermann, M. N.; Powell, R. E. Air Oxidation of Hydroxylamine-*N*-sulfonate. *Inorg. Chem.* **1967**, *6*, 1718–1720.

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(21) (a) Hantzsch, A. Salts of Nitrosylsulfuric Acid (in German). *Ber. Dtsch. Chem. Ges.* **1894**, *27*, 3264–3273. (b) Divers, E.; Haga, T. The Constitution of the Nitrososulphates. *J. Chem. Soc.* **1895**, *67*, 1098–1104.

the findings of the present investigation support the conclusion that nucleophile/•NO complexes can serve as reliable vehicles for spontaneous but predictable and controllable delivery of nitric oxide in a biological system.

A similar correlation has been seen between $1/EC_{50}$ for soluble guanylate cyclase activation and the rate of nitric oxide production from various sydnoneimine and nitroglycerin derivatives.[22] The latter compounds differ from ours in that they require redox activation[23,24] while the nucleophile/•NO complexes described herein generate nitric oxide at a predictable rate purely spontaneously. Thus the nucleophile/•NO complexes studied here have the advantages as biological research tools that they are stable as solids, deliver nitric oxide in a biologically usable form at a predictable rate, and do not require activation by electron transfer.

The only other vasodilators that to our knowledge have been confirmed to release •NO without obligatory redox activation are the *S*-nitroso thiols.[24-26] Evidence has recently been presented that the endothelium-derived relaxing factor is actually one or more *S*-nitroso compounds having lifetimes similar to that of free •NO in the physiological milieu,[25] but at least a portion of these compounds' vasoactivity appears to result from activation by tissue components.[26] Since the strong reactivity–potency correlation shown in Figure 4 suggests that the action of the nucleophile/•NO complexes is entirely metabolism-independent, the latter compounds may offer a convenient means of experimentally distingishing between the *S*-nitroso thiols' spontaneous versus catalyzed modes of action.

The present data also show that the biological effects of the nucleophile/•NO complexes can be potent. As measured via the ability to induce relaxation in aortic rings, the most rapid and extensive nitric oxide progenitors studied here (**1**, **2**, and **4**) were at least as potent as the positive controls sodium nitroprusside (EC_{50} = 2.0 μM) and glyceryl trinitrate (EC_{50} > 10 μM).

The results suggest that complexes of nitric oxide with nucleophiles may be of considerable value in biomedical research. A vast number of specific compounds having the requisite $XN(O^-)N{=}O$ structure should be isolable, and they can be expected to cover a wide range of decomposition rates, product profiles, and extents of •NO release. Thus, they could be useful in many applications requiring the controlled generation of nitric oxide in solution, and may provide a basis for rational drug design.

Significance

Nitric oxide in its pure form is a highly reactive gas having limited solubility in aqueous media,[27] making it difficult to introduce reliably into most biological systems without premature decomposition. In many cases, these difficulties can be overcome by administering •NO pharmacologically in prodrug form. For example, the widely used nitrovasodilators glyceryl trinitrate and sodium nitroprusside are relatively stable but release nitric oxide on redox activation.[24,28] While this feature is a tremendous advantage in many applications, it can also be a liability, as in the development of tolerance to glyceryl trinitrate via the exhaustion of the relevant enzyme/cofactor system[29] and toxicity from metabolically produced cyanide during prolonged administration of nitroprusside.[30]

We believe that compounds **1**–**5** and related nucleophile/•NO complexes offer a promising approach to circumventing these disadvantages. They stabilize •NO during storage in a solid form that is, in general, highly soluble in the aqueous milieu. Moreover, the rate at which •NO is generated upon introduction into the biological system can be adjusted reliably over a wide range with judicious choice of the carrier nucleophile. Nontoxic species (e.g., water from **4**) and/or a vehicle with potentially beneficial properties in its own right (such as spermine from **3**) can be chosen as nucleophiles. It is hoped that extensive structure–activity and –reactivity studies now in progress will lead to attractive candidates for the treatment of hypertension and related cardiovascular disorders, as well as for application to a variety of other problems in biomedical research.

Experimental Section

Compounds **1**, **2**, **4**, and **5** were synthesized according to literature procedures (refs 4, 5, 14, and 31, respectively), except that in the preparation of **2**, sodium methoxide (rather than ethoxide) was used to neutralize the intermediate isopropylammonium salt. The purities of **1** and **2** were checked by nuclear magnetic resonance (NMR) spectrometry, with only a single ethyl or isopropyl group, respectively, in the proton spectrum. Compound **4** gave

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the expected ultraviolet spectrum, λ_{max} 248 nm and ϵ_{max} 8.3×10^3 M^{-1} cm^{-1} in 0.1 M sodium hydroxide [lit.[14] λ_{max} 248 nm (dilute alkali) and ϵ_{max} 8.3×10^3 M^{-1} cm^{-1}], with no evidence of nitrite contamination[32] at 357 nm; compound 4 was recrystallized from aqueous ethanol before use, a procedure shown to produce the monohydrate.[33] The ultraviolet properties of 5 (λ_{max} 259 nm, ϵ_{max} 7.1×10^3 M^{-1} cm^{-1}) compared favorably with literature values[18] for the dipotassium salt (λ_{max} 258 nm, ϵ_{max} 7.1×10^3 M^{-1} cm^{-1}). Spermine was purchased from Sigma Chemical Company, St. Louis, MO. Nitric oxide used for syntheses was commercial grade (Matheson Gas Products, East Rutherford, NJ). The primary reference standard (Matheson) used in quantifying •NO was 52 ppm nitric oxide (containing 1 ppm nitrogen dioxide) in helium. Infrared spectra were determined with a Perkin-Elmer Model 467 grating infrared spectrophotometer. The ^1H NMR spectra were determined at 200 MHz with a Varian Model XL-200 NMR spectrometer and the ^{13}C NMR spectra were recorded using the same instrument at 50 MHz. The chemical shifts are expressed in δ values (ppm) relative to sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$ as internal standard. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

Preparation of Spermine Bis(nitric oxide) Adduct (3). A solution of spermine (4.70 g, 23.2 mmol) in 300 mL of dry tetrahydrofuran was deoxygenated with nitrogen and placed in a glass bottle on a standard Model 3911 hydrogenation apparatus (Parr Instrument Company, Moline, IL). The solution was maintained at room temperature (ca. 22 °C) and •NO gas was admitted to a pressure of 5 atm. Within 20 min the solution became cloudy at the surface and solid began forming. The pressure was kept at 70–75 psig by occasional addition of •NO, and the solution was allowed to stand without shaking for 5 days, whereupon the excess pressure was vented and the mixture was flushed with nitrogen for 5 min. The voluminous white precipitate was filtered, washed with ether, and dried in vacuo for 2 h to afford 2.11 g of product as a white, amorphous powder in 35% yield: mp 105–107 °C dec; IR (KBr pellet) 2200–3200 (i, br), 1580 (m, br), 1470 (m, br), 1380 (m), 1270 (w), 1150 (i, br), 940 (m, br) cm^{-1}; ^1H NMR (D_2O) δ 1.38 (2 H, m), 1.58 (4 H, m), 1.75 (2 H, m), 2.70–2.85 (8 H, m), 2.96 (4 H, m); ^{13}C NMR (D_2O) δ 26.35, 27.68, 30.00, 31.59, 40.97, 41.04, 48.56, 50.67, 54.37, 56.44. Anal. ($C_{10}H_{26}N_6O_2 \cdot H_2O$) C, N; H, calcd, 10.07; found, 8.94.

Ultraviolet Studies. The ultraviolet data were collected using a Hewlett-Packard Model 8451 diode-array spectrophotometer. Extinction coefficients were determined by dissolving a known amount of test substance in 0.01 M potassium hydroxide and then diluting with a large excess of buffer and recording the spectrum within 1–2 s of dilution. Spectra determined intermittently thereafter in the thermostated (37 °C) cuvette were used to calculate *k* values [computed from the slopes of the ln (absorbance − absorbance$_\infty$) versus time plots].

Chemiluminescence Measurements. Nitric oxide was quantified with a Thermal Energy Analyzer (Model 502LC, Thermedics, Inc., Woburn, MA). A thermostated (37 °C) reactor vessel similar in design to that previously described by Feelisch and Noack[34] but with additional dry ice/methanol traps was flushed with helium under vacuum for 10 min to remove residual oxygen and nitric oxide. The sample to be studied was then introduced into the apparatus by injecting 0.05 mL of freshly dissolved material in 0.01 M potassium hydroxide via a septum. Decomposition reactions, begun within 2 min of dissolution, were initiated by the addition of 0.95 mL of 0.1 M pH 7.4 phosphate buffer. Gaseous products purged from solution were flushed continuously into the detector with helium. Data were digitized using a Hewlett-Packard integrator. Areas of observed peaks were converted to moles by comparison with that of an •NO calibrant gas.

Vasoactivity Determinations. Freshly isolated rings of rabbit thoracic aorta were attached to force transducer tensiometers and equilibrated in a series of chambers containing 50 mL of modified Krebs buffer and purged with 95:5 oxygen/carbon dioxide to maintain pH at 7.4 at 37 °C. The buffer was composed of 120 mM sodium chloride, 25 mM sodium bicarbonate, 4.7 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM magnesium sulfate, 1.2 mM potassium dihydrogen phosphate, 38 μM EDTA, 20 μM cocaine, and 2 μM propanolol. Each ring was constricted with the EC_{50} dose of norepinephrine for that ring (typically 0.3 μM). The test substance, freshly dissolved in 10 mM sodium hydroxide (for 1–5) or distilled water (for sodium nitroprusside), was then added to the chamber at the lowest dose level (usually 1 nM). The change in force exerted by the aortic strip was charted on a four-channel Grass recorder. The degree of the arterial relaxation was computed from the maximum pen deflection and expressed as a percentage of the tension generated during norepinephrine constriction. Upon reaching the maximum deflection, the next higher dose was added and the measurement was repeated. This process was reiterated for successively higher concentrations, providing the data illustrated in Figure 3. As soon as the data for one compound were collected, the rings were reequilibrated in fresh buffer, and the process was repeated with the next compound. The biological data of Table I were collected on two consecutive days. Four rings from the same rabbit were used in parallel the first day, with four different compounds tested in each round. A similar procedure with three rings from a different rabbit was used the second day. Replicates were generally performed on different rings and at different times throughout the 2-day period so as to minimize systematic errors arising from interring variability and ring age.

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Supplementary Material Available: Derivation of eq 4 and complete dose–response curves for all compounds whose vasorelaxant effects were quantified in the present simultaneous comparison, including compounds 1–5, •NO, sodium nitroprusside, and glyceryl trinitrate (4 pages). Ordering information is given on any current masthead page.

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