Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 9, Issue of March 4, pp. 6282–6285, 1994

Caged Nitric Oxide

STABLE ORGANIC MOLECULES FROM WHICH NITRIC OXIDE CAN BE PHOTORELEASED*

(Received for publication, December 10, 1993)

Lewis R. Makings and Roger Y. Tsien‡

From the Howard Hughes Medical Institute and Departments of Pharmacology and Chemistry, University of California at San Diego, La Jolla, California 92093-0647

We report the synthesis and testing of a series of "caged" nitric oxide compounds that are stable indefinitely in oxygen-containing solutions until photolyzed by ultraviolet irradiation, whereupon they release nitric oxide (NO) with quantum yields of Δ5% for 3a (CNO-1) and Δ2% for compounds 3b-e (CNO2–5). After a flash, NO release is complete within 5 ms, so that precise temporal control of NO release is possible. NO donor 3d (CNO-4) includes two carboxylate negative charges at physiological pH, which reduce membrane permeability and enable photolytic generation of NO to be selectively confined to either extracellular or intracellular compartments. Esterification of these carboxyls with acetoxymethyl groups produces 3e (CNO-5), which is membrane-permeant and intracellularly hydrolyzable. Therefore, large populations of intact cells can be conveniently intracellularly loaded with "caged" NO donor 3d by incubation with 3e (CNO-5). The biological efficacy of these NO donors and their absolute dependence on UV-irradiation was demonstrated by inhibition of thrombin-stimulated platelet aggregation. Extracellular hemoglobin blocked the effects of NO generated outside but not inside platelets, verifying the sidedness of the NO donors and the limited spatial range of NO action. These molecules should permit precise spatial, temporal, and concentration control of NO release for investigation of its important biological functions.

Nitric oxide (NO) has recently been implicated in a wide variety of bioregulatory and pathophysiological processes (1–3), including a possible role as an important messenger in synaptic plasticity (4–6). Disentanglement of physiologically relevant from pathological or artifactual actions of NO has been hindered by experimentalists' inability to deliver NO at controllable concentrations with spatial and temporal precision comparable with endogenous NO generation (7, 8). NO itself is a hard-to-handle toxic reactive gas. Commonly used NO donors each suffer from one or more limitations that restrict mechanistic investigations. For example, sodium nitroprusside requires redox activation before NO can be released (9) and also produces toxic cyanide. The NO donor 3-morpholinosydnonimine (SIN-1) requires oxidation by oxygen, so that superoxide is produced together with the NO (10). These and many other NO donors release NO relatively slowly, a property that can be desirable in some applications. However, for investigations into the kinetics of NO responses, especially in neurobiology, localized NO release in a few milliseconds could be very useful. To fill this need, we report photochemically triggered NO donors that can be tailored either to permeate cell membranes or to remain excluded from cells or to become specifically trapped in their intracellular spaces. These relatively small molecules are stable and biologically inert until photolyzed, whereupon NO is released in less than 5 ms.

* This work was supported by the Howard Hughes Medical Institute and National Institutes of Health Grant NS27177. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Inquiries about purchasing the compound should be directed to Alexis Corp., 3-morpholinosydnonimine Cornerstone Court East, Suites 102–104, San Diego, California 92121-4727.

EXPERIMENTAL PROCEDURES

Synthesis—The structures and synthetic routes are shown in Fig. 1. Reagents were from Aldrich. Solvents (high pressure liquid chromatography grade) were dried over an activated molecular sieve (3 Å). Nitric oxide (99.0%) was from Matheson Gas Products. The $Et_2N(N_2O_2)Na$ (11, 12), $Ru(NO)Cl_3$ (13), and 4,5-dimethoxy-2-nitrobenzyl bromide **2b** (14) were prepared as described previously. The complete synthetic details for the preparation of **2c** will appear elsewhere. Briefly, the dihydroxybenzaldehyde was alkylated in dimethylformamide using ethyl bromoacetate and K_2CO_3, then nitrated with HNO_3. The aldehyde functionality was reduced to the alcohol with $NaBH_4$ and then converted to the bromide with PBr_3 in a manner similar to that reported by Wilcox *et al.* (14) for **2b**. The synthesis of **3a**, **3b**, and **3c** was carried out using the general synthetic procedure (see Fig. 1) described in detail for **3c**.

1-[(2'-Nitrophenyl)methoxy]-2-oxo-3,3-diethyl-1-triazene **(3a)**—After purification 72 mg (48% yield) of **3a** was obtained as a colorless oil: ^1H NMR ($CDCl_3$) δ 1.06 (t, 6H, NCH_2CH_3), 3.13 (q, 4H, NCH_2CH_3), 5.72 (s, 2H, OCH_2Ar), 7.48 (m, 1H, ArH), 7.64 (m, 2H, ArH), 8.15 (d, 1H, ArH). MS(FAB)[1] *m/z* $(M+H)^+$ 269.

1-[(4',5'-Dimethoxy-2'-nitrophenyl)methoxy]-2-oxo-3,3-diethyl-1-triazene **(3b)**—After purification 112 mg (34% yield) of **3b** was obtained as a white solid, melting point 106–107 °C: ^1H NMR ($CDCl_3$) δ 1.08 (t, 6H, NCH_2CH_3), 3.16 (q, 4H, NCH_2CH_3), 3.95 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 5.73 (s, 2H, OCH_2Ar), 7.11 (s, 1H, ArH), 7.74 (s, 1H, ArH). MS(FAB) *m/z* $(M+H)^+$ 343.

1-[(4',5'-Bis(ethoxycarbonylmethoxy)-2'-nitrophenyl)methoxy]-2-oxo-3,3-diethyl-1-triazene **(3c)**—To a solution of $Et_2N(N_2O_2)Na$ (**1**, 75 mg, 0.48 mmol) in 1 ml of dry dimethylformamide was added **2c** (170 mg, 0.4 mmol). The reaction was stirred at room temperature under an argon atmosphere. After 1 h, 20 ml of toluene was added, and the mixture was washed with water (3 × 20 ml). The organic phase was dried with sodium sulfate, filtered, and evaporated to give the crude product as a yellow oil, which was purified by chromatography on silica gel eluting with ethyl acetate/hexane (1:3, v/v) to give 72 mg (38% yield) of **3c** as a white solid, melting point 80–82 °C: ^1H NMR ($CDCl_3$) δ 1.08 (t, 6H, NCH_2CH_3), 1.31 (2t, 6H, OCH_2CH_3), 3.16 (q, 4H, NCH_2CH_3), 4.28 (2q, 4H, OCH_2CH_3), 4.77 (s, 2H, CH_2OAr), 4.78 (s, 2H, CH_2OAr), 5.68 (s, 2H, $NOCH_2Ar$), 7.07 (s, 1H, ArH), 7.73 (s, 1H, ArH). MS(FAB) *m/z* $(M+H)^+$ 373.

1-[(4',5'-Bis(carboxymethoxy)-2'-nitrophenyl)methoxy]-2-oxo-3,3-diethyl-1-triazene $(K^+)_2$ **(3d)**—Ester **3c** (27 mg, 55 μmol) was dissolved in 300 μl of dioxane, to which 500 μl of methanol and 60 μl of 1 M aqueous KOH were added. An additional 60 μl of base was added after 1 h at room temperature. After a total reaction time of 3 h the solution was diluted with 800 μl of dioxane. The precipitated product was isolated by centrifugation, triturated with ether, and dried under vacuum to give 25 mg (93% yield) of **3d** as the potassium salt, mp 300–305 °C decomposes: ^1H NMR (D_2O) δ 1.06 (t, 6H, NCH_2CH_3), 3.22 (q, 4H, NCH_2CH_3), 4.76 (s, 2H, CH_2OAr), 4.79 (s, 2H, CH_2OAr), 5.85 (s, 2H, $NOCH_2Ar$), 7.14 (s, 1H, ArH), 7.83 (s, 1H, ArH). MS(FAB) *m/z* $(M^{2-}+K^+)$ 453.

1-[(4',5'-Bis(carboxymethoxy)-2'-nitrophenyl)methoxy]-2-oxo-3,3-diethyl-1-triazene Acetoxymethyl Ester **(3e)**—The potassium salt **3d** (5

[1] The abbreviations used are: MS, mass spectroscopy; FAB, fast atom bombardment.

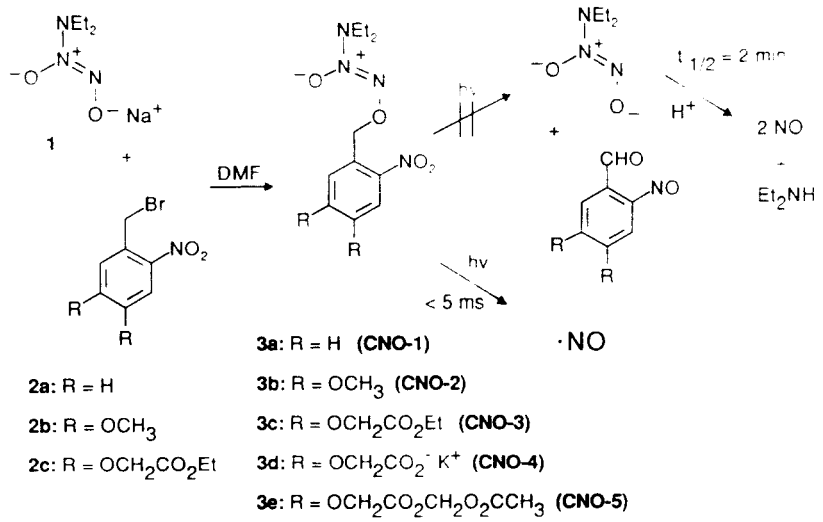

FIG. 1. **Structures of the NO donors, synthetic route, and proposed photolytic products.**

mg, 10 μmol) was dissolved in 150 μl of water and cooled to 5 °C. Addition of 25 μl of 1 M HCl precipitated the free acid, another 150 μl of water was added, and the precipitate was pelleted by centrifugation. The precipitate was washed with cold water, collected, and dried under vacuum to give 3 mg (7.3 μmol) of the free acid. This material was suspended in 200 μl of dry CH_2Cl_2, and 30 μl of diisopropylethylamine and 20 μl (200 μmol) of bromomethyl acetate were added. The now homogeneous solution was left at room temperature under argon. After 16 h, the CH_2Cl_2 was evaporated and the residue partitioned between toluene and water. The toluene was dried with Na_2SO_4, filtered, and evaporated to give an oil, which was purified by chromatography on silica gel eluting with ethyl acetate/hexane (2:1) to give 2.6 mg (66% yield) of **3d** as an oil: ^1H NMR ($CDCl_3$) δ 1.08 (t, 6H, NCH_2CH_3), 2.14 (2s, 6H, O_2CCH_3), 3.18 (q, 4H, NCH_2CH_3), 4.83 (s, 2H, CH_2OAr), 4.84 (s, 2H, CH_2OAr), 5.67 (s, 2H, $NOCH_2Ar$), 5.85 (s 4H, $CO_2CH_2O_2C$), 7.10 (s, 1H, ArH), 7.74 (s, 1H, ArH). MS(FAB) m/z $(M+H)^+$ 561.

Hemoglobin Preparation—Human hemoglobin (HbO_2) was obtained as described previously (15) and used within 1 week of isolation. Deoxyhemoglobin (Hb) was obtained by diluting HbO_2 into degassed 50 mM phosphate buffer (pH 7.0) containing the NO donor. A surface-agitating argon stream was passed over the solution until >95% Hb was obtained as determined by the visible absorbance spectrum. Solutions containing Hb were maintained under Ar.

Flash Photolysis Kinetics—The millisecond time resolution flash photolysis experiments were performed in the following manner. The excitation flash (200 joules discharge energy, 300 μs full width at half-maximum) was produced by a Strobex 238 power supply and 278 xenon flash lamp (Chadwick-Helmuth Co., El Monte, CA) equipped with a 340-nm long-pass filter. The continuous probe light was orthogonal to the excitation flash and was generated by a 12-V, 60-watt tungsten lamp equipped with a 400-nm long-pass filter. The transmitted light was passed through the monochromator and emission detection system of a K2 fluorometer (ISS, Champaign, IL). The maximum sample rate of the fluorometer was 200 s^{-1}. Faster instrumental resolution might have been obtained with alternative electronics but was not felt to be biologically essential. Also, the reaction kinetics of Hb with NO would have become limiting.

Platelet Isolation and Aggregation Measurements—The platelets were obtained from the blood of healthy human donors who had not ingested aspirin for at least 2 weeks. The washed platelets were prepared in a similar manner to that described previously (16) with the exclusion of the quin-2 and prostacyclin additions. Platelet aggregation was monitored in 0.5-ml volumes by continuous recording of light transmission in an aggregometer (Chrono-Log Co., Haverston, PA). The platelets were incubated at 37 °C with stirring (1100 rpm) for 3 min prior to the addition of the NO donors. In a typical experiment a 10 mM stock solution of the NO donor was added to the suspension of platelets so as to produce the desired final concentration. The solution was then incubated in the dark with stirring at 37 °C for 10 min in the aggregation tubes. The solution was irradiated by exposing the tubes to filtered 365-nm light (≈6 milliwatts/cm^2) from a mercury lamp (TC-365A Transilluminator, Spectronics Co., Westbury, NY) for various time periods. In the dose-response experiments, the NO donor was rapidly (<30 s) and completely photolyzed by exposing the tubes to 10 flashes each of 200 joules discharge energy from the xenon flash lamp. The sample tube was then returned to the aggregometer, and a threshold dose (40–80 milliunits/ml) of thrombin was added to induce aggregation. The recorded change in light transmission relative to that obtained with the same concentration of thrombin in the absence of any NO donor was then used to calculate values for percent inhibition of platelet aggregation. Error estimates are standard deviations. In experiments where oxyhemoglobin was used as an extracellular NO trap, the HbO_2 was added producing a final concentration of 20 μM just prior to the photolysis of the NO donor. Control experiments involving photolysis of the platelet suspension with and without hemoglobin in the absence of NO donors caused less than 5% inhibition relative to unphotolyzed controls. Similarly, incubation of the platelets with the NO donors in the dark for 10 min under the experimental conditions described above also resulted in less than 5% inhibition of thrombin-induced platelet aggregation.

RESULTS AND DISCUSSION

Masking of biologically active molecules with photoremovable 2-nitrobenzyl groups is a valuable technique used in many areas of research (17, 18). Keefer and colleagues (19, 20) had already introduced $Et_2N(N_2O_2)^-$ (**1**) and various analogs as molecules that spontaneously release NO in neutral aqueous medium, whereas alkylation of the 1-oxygen prevents such decomposition (21). We therefore decided to alkylate **1** with photolabile 2-nitrobenzyl moieties such as shown in Fig. 1. The resulting NO donors **CNO-1** through **CNO-5** were indeed completely stable in oxygenated aqueous solutions until photolysis, whereupon they released NO. The formation of NO from compounds **3a–e** was determined spectrophotometrically using the well known NO trap deoxyhemoglobin, Hb (22, 23). As shown in Fig. 2, irradiation of **3b** at 365 nm in the presence of Hb converted the absorbance spectrum of Hb to the spectrum characteristic of nitrosylhemoglobin (HbNO) (24). Light-induced NO formation was also verified in oxygenated media by conversion of oxyhemoglobin to methemoglobin (25) and production in protein-free media of nitrite, which was determined spectrophotometrically by the Griess reaction (26). In spite of the harsh conditions used in the latter method (pH 1 for 30 min), nitrite was only detected in solutions containing the NO donors that had been previously photolyzed. The quantum yields for the disappearance of **3a–e** and production of NO are given in Table I. Although the yields are modest, they are comparable with $Ru(NO)Cl_3$, which is the only other NO donor so far reported to be stable in the dark yet rapidly releasing NO upon irradiation (27).

Keefer and co-workers (19) have extensively studied the formation of NO from the spontaneous decomposition of **1**, which is easily followed by the loss of its characteristic absorbance (λ_{max} = 248 nm, $\epsilon = 8.3 \times 10^3$ M^{-1} cm^{-1}) and has a half-life of 2.1 min at pH 7.4, 37 °C (19). Therefore, upon photolysis of com-

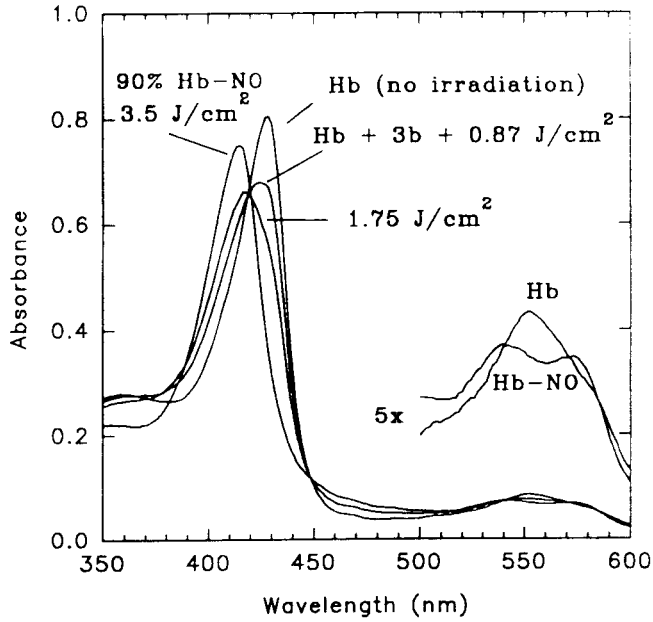

FIG. 2. **Absorbance spectra change of deoxyhemoglobin (Hb) (6 μM in hemes) plus 10 μM 3b in 50 mM phosphate buffer (pH 7.0, 22 °C) before and after irradiation with a 365 nm Hg lamp to give the indicated incident fluxes.** The *inset* at *lower right* shows the Q-bands at 0 and 90% conversion amplified 5-fold for clarity.

TABLE I

*Quantum yields (Φ) for photolysis of compounds **3a–3** at 365 nm*

Compound 3	ϵ_{365}	Φ_{dis}[a]	Φ_{NO}[b]
	$M^{-1}\ cm^{-1}$		
3a	300	0.09_6	0.05_2
3b	3900	0.03_2	0.01_9
3c	2100	0.04_6	0.02_4
3d	3700	0.03_1	0.01_8
3e	2100		0.02_2

[a] Quantum efficiency for loss of starting material measured by absorbance changes upon photolysis of 25 μM solutions of **3a–d** in 50 mM phosphate buffer (pH 7.0, 22 °C) with 365 nm light of measured irradiance (41, 42).

[b] Quantum efficiency measured by spectrophotometrically determining the amount of Hb converted to HbNO upon irradiating 10 μM **3a–e** and 6 μM Hb in 50 mM phosphate buffer (pH 7.0, 22 °C) with 365 nm light of measured irradiance.

pounds **3a–e** with a brief (<1 ms) flash of light, one might have expected **1** to be immediately regenerated, followed over the next few minutes by a slow decrease in the absorbance at 248 nm as **1** decomposed to diethylamine and NO. However, the flash-induced decrease in absorbance around 250 nm (from the loss of the 2-nitrobenzyl chromophore as well as the triazene chromophore) was actually complete in less than 1 s rather than minutes. The kinetics of the release of NO from **3b** were then investigated using Hb, which binds NO rapidly. Upon excitation of a solution containing **3b** and Hb with a single pulse (300 μs full width at half-maximum) from a xenon flash lamp, the conversion of Hb to HbNO was complete within a single data point (≤5 ms) after the flash artifact as shown by the increase in transmitted light intensity at 430 nm (Fig. 3*A*). This time resolution is close to the limiting value set by the second-order rate constant for NO binding to Hb, $1.8 \times 10^7\ M^{-1}\ s^{-1}$ (28). Control experiments (traces *B* and *C*) indicated that the flash lamp alone or the by-products from the uncaging reaction (mimicked by photolysis of 4,5-dimethoxy-2-nitrobenzyl alcohol) could not account for the loss in Hb absorbance at 430 nm seen in trace *A*. In similar experiments (data not shown) the rapid release of NO from **3b** and **3d** was detected using the NO traps HbO_2 and microperoxidase (MP-11) (29).

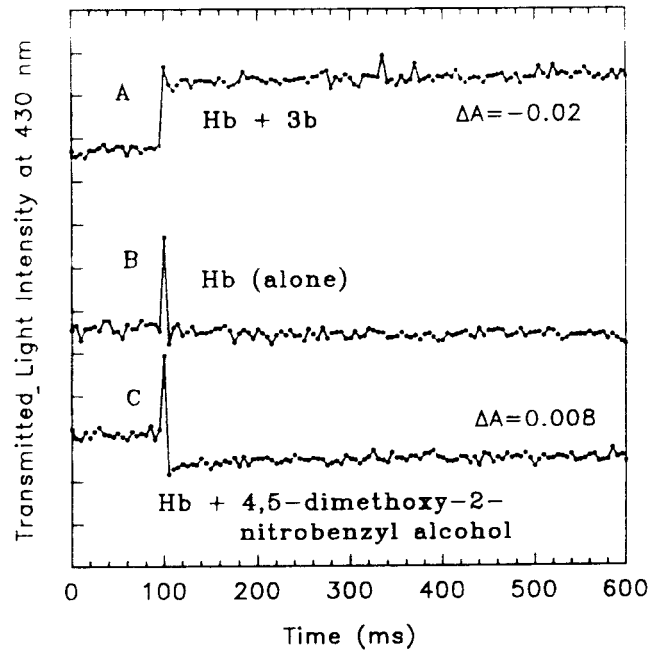

FIG. 3. **Change in transmitted light intensity at 430 nm when Hb (8 μM in hemes) in 50 mM phosphate buffer (pH 7.0, 22 °C) is irradiated with a single pulse (300 μs full width at half-maximum) from a filtered (λ > 340 nm) xenon flash lamp.** *A*, in the presence of 100 μM **3b**; *B*, in the absence of **3b**. *C*, in the presence of 100 μM 4,5-dimethoxy-2-nitrobenzyl alcohol (the absorbance change is due to the photoproducts produced with Φ = 0.10 (41) and is in the same direction and amplitude in the presence and absence of Hb).

One possible rationalization for the rapidity of NO release is that the breakdown of **1** is known to be acid-catalyzed (19), perhaps by protonation of the diethylamino group, while one of the first steps in the photolysis of 2-nitrobenzyl compounds is ejection of one of the benzylic protons (18). If that proton were picked up by the diethylamine, then fragmentation to NO could be nearly instantaneous.

Biological efficacy of the photoreleasable NO donors was tested on human platelets, because inhibition of thrombin-stimulated platelet aggregation is a well known biological assay for NO (30–32), probably mediated by stimulation of guanylate cyclase. All three NO donors were without effect in the dark but could completely inhibit aggregation when photolyzed. Compounds **3b** and **3d** had similar inhibitory potencies that demonstrated dependences on concentration and light exposure, as expected for photoreleasable NO donors. Dose-response measurements obtained from the rapid and complete photolysis of **3d** gave a value of ≈1 μM as the concentration for 50% inhibition of platelet aggregation. The permeability of **3b, 3d**, and **3e** across platelet membranes was investigated using HbO_2 as an extracellular NO trap (Table II). The two negative charges on **3d** seemed to prevent it from crossing cell membranes readily, since extracellular HbO_2 completely prevented any inhibition due to photolysis of extracellular **3d**. Acetoxymethyl esters such as **3e** are well known to cross plasma membranes and hydrolyze intracellularly, specifically trapping the anions (in this case **3d**) inside cells (33, 34). After such trapping extracellular HbO_2 was completely ineffective. This result not only confirms that the NO donor was inside the cells but also suggests that the photolytically generated NO binds to guanylate cyclase faster than it can diffuse over a distance of a few microns or so. As expected, uncharged **3b** seemed to equilibrate across the plasma membrane, since extracellular HbO_2 only partially blocked the NO response.

Other compounds that "cage" NO have been proposed, *e.g.* $Ru(NO)Cl_3$ (27, 35) and "Roussin's Black Salt," an iron-sulfur-

TABLE II
Efficacy of NO photolytically generated outside versus inside platelets

NO source[a]	Aggregation inducible by thrombin[b]		n^c
	Without HbO_2	With extracellular HbO_2	
	%		
3b (CNO-2), 40 μM	2 ± 3	65 ± 4	4
3d (CNO-4), 40 μM	4 ± 4	94 ± 3	4
3e (CNO-5), 20 μM	2 ± 3	6 ± 4	3
$Ru(NO)Cl_3$, 40 μM	95 ± 4		4

[a] NO donors were added at the indicated concentrations to the platelet suspension and exposed to 365 nm for 4 min at an intensity sufficient to photolyze about 50% of the NO donor.

[b] Thrombin was 40–80 milliunits/ml, HbO_2 when present was 20 μM in hemes. Error estimates on percent aggregations are standard deviations.

[c] Number of experiments with and without HbO_2, except for $Ru(NO)Cl_3$, which was not tested with HbO_2.

nitrosyl complex (36, 37). Roussin's Black Salt has the drawback that it spontaneously releases NO at a significant rate even in the dark (38, 39). Both compounds might be metabolized inside cells and could also release potentially toxic heavy metals upon photolysis. In our hands, $Ru(NO)Cl_3$ was very effective at producing HbNO upon photolysis; however, it failed to inhibit platelet aggregation when tested in the same way and at the same concentrations as **3b, 3d,** and **3e** (Table II). Recently, the light-induced decomposition of phenyl-*tert*-butylnitrone was reported to form NO (40). However, the quantum yield and rate of NO formation were not given, and considering the multiple photon and oxidation steps proposed for the decomposition, the yield appears to be low and the rate of release is probably slow. In addition, the authors suggest that under biological conditions phenyl-*tert*-butylnitrone may form NO without light.

The spatial, temporal, and amplitude control made possible by photoactivation should make **3d–e** and further analogues valuable for probing the wide variety of biological functions of NO. The site of NO generation should be controllable by putting **3d** in the extracellular medium or into specific cells by microinjection, whole-cell patch clamp, or acetoxymethyl ester (**3e**) loading. Further localization should be possible by focused illumination. Such control may be particularly helpful in elucidating potential roles for NO in the brain, where the biological effects of NO may depend strongly on exactly how much is delivered and to which cells.

REFERENCES

1. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142
2. McCall, T., and Vallance, P. (1992) *Trends Pharmacol. Sci.* **13,** 1–6
3. Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992) *Science* **258,** 1898–1902
4. Bliss, T. V. P., and Collingridge, G. L. (1993) *Nature* **361,** 31–39
5. Bredt, D. S., and Snyder, S. H. (1992) *Neuron* **8,** 3–11
6. Garthwaite, J. (1991) *Trends Neurosci.* **14,** 60–67
7. Izumi, Y., Clifford, D. B., and Zorumski, C. F. (1992) *Science* **257,** 1273–1276
8. Shibuki, K., and Okada, D. (1991) *Nature* **349,** 326–328
9. Smith, R. P., Wilcox, D. E., Kruszyna, H., and Kruszyna, R. (1990) in *Biological Reactive Intermediates IV* (Witmer, C. M., ed) pp. 365–369, Plenum Press, New York
10. Feelisch, M., Ostrowski, J., and Noack, E. (1989) *J. Cardiovasc. Pharmacol.* **14,** Suppl. 11, S13–S22
11. Drago, R. S., and Karstetter, B. R. (1961) *J. Am. Chem. Soc.* **83,** 1819–1822
12. Drago, R. S., and Paulik, F. E. (1960) *J. Am. Chem. Soc.* **82,** 96–98
13. Muller, J. G., and Takeuchi, K. J. (1990) *Inorg. Chem.* **29,** 2185–2188
14. Wilcox, M., Viola, R. W., Johnson, K. W., Billington, A. P., Carpenter, B. K., McCray, J. A., Guzikowski, A. P., and Hess, G. P. (1990) *J. Org. Chem.* **55,** 1585–1589
15. Antonini, E., and Brunori, M. (1971) in *Hemoglobin and Myoglobin in Their Reactions with Ligands* (Neuberger, A., and Tatum, E. L., eds) pp. 1–12, North-Holland, Amsterdam
16. Hallam, T. J., Sanchez, A., and Rink, T. J. (1984) *Biochem. J.* **218,** 819–827
17. Adams, S. R., and Tsien, R. Y. (1993) *Annu. Rev. Physiol.* **55,** 755–784
18. McCray, J. A., and Trentham, D. R. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18,** 239–270
19. Maragosa, C. M., Morley, D., Wink, D. A., Dunams, T. M., Saavedra, J. E., Hoffman, A., Bove, A. A., Issac, L., Hrabie, J. A., and Keefer, L. K. (1991) *J. Med. Chem.* **34,** 3242–3247
20. Hrabie, J. A., Klose, J. R., Wink, D. A., and Keefer, L. K. (1993) *J. Org. Chem.* **58,** 1472–1476
21. Saavedra, J. E., Dunams, T. M., Flippen-Anderson, J. L., and Keefer, L. K. (1992) *J. Org. Chem.* **57,** 6134–6138
22. Cassoly, R., and Gibson, Q. H. (1975) *J. Mol. Biol.* **91,** 301–313
23. Doyle, M. P., and Mahapatro, S. N. (1984) *J. Am. Chem. Soc.* **106,** 3678–3679
24. Dilorio, E. E. (1981) *Methods Enzymol.* **76,** 57–72
25. Noack, E., Kubitzek, D., and Kojda, G. (1992) in *NeuroProtocols, Detecting Nitrosyl Products* (Bates, J. N., ed) pp. 133–139, Academic Press, New York
26. Tracey, W. R. (1992) in *NeuroProtocols, Detecting Nitrosyl Products* (Bates, J. N., ed) pp. 125–131, Academic Press, New York
27. Carter, T. D., Bettache, N., Ogden, D., and Trentham, D. R. (1993) *J. Physiol. (Lond.)* **467,** 165P (abstr.)
28. Rose, E. J., and Hoffman, B. M. (1983) *J. Am. Chem. Soc.* **105,** 2866–2873
29. Sharma, V. S., Isaacson, R. A., John, M. E., Waterman, M. R., and Chevion, M. (1983) *Biochemistry* **22,** 3897–3902
30. Ivanova, K., Schaefer, M., Drummer, C., and Gerzer, R. (1993) *Eur. J. Pharmacol.* **244,** 37–47
31. Salvemini, D., Radziszewski, W., Korbut, R., and Vane, J. (1990) *Br. J. Pharmacol.* **101,** 991–995
32. Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987) *Br. J. Pharmacol.* **92,** 181–187
33. Tsien, R. Y. (1989) *Annu. Rev. Neurosci.* **12,** 227–253
34. Tsien, R. Y. (1981) *Nature* **290,** 527–528
35. Williams, J. H., Bettache, N., Trentham, D. R., and Bliss, T. V. P. (1993) *J. Physiol. (Lond.)* **467,** 166P (abstr.)
36. Boulton, C. L., Irving, A. J., Collingridge, G. L., and Garthwaite, J. (1993) *J. Physiol. (Lond.)* **467,** 181P (abstr.)
37. Flitney, F. W., Megson, I. L., Thomson, J. L. M., and Kennovin, G. D. (1993) *J. Physiol. (Lond.)* **459,** 90P (abstr.)
38. Flitney, F. W., Megson, I. L., and Butler, A. R. (1993) *J. Physiol. (Lond.)* **459,** 89P (abstr.)
39. Flitney, F. W., Megson, I. L., Flitney, D. E., and Butler, A. R. (1992) *Br. J. Pharmacol.* **107,** 842–848
40. Chamulitrat, W., Jordan, S. J., Mason, R. P., Saito, K., and Cutler, R. G. (1993) *J. Biol. Chem.* **268,** 11520–11527
41. Adams, S. R., Kao, J. P. Y., and Tsien, R. Y. (1989) *J. Am. Chem. Soc.* **111,** 7957–7968
42. Tsien, R. Y., and Zucker, R. S. (1986) *Biophys. J.* **50,** 843–853