analogy with other neurons (*11*), the first step is the action potential generated in the initial segment, and the second is that generated in the soma-dendritic membrane. The records in Fig. 1E demonstrate that the presence or absence of an impulse in the initial segment has little effect on the size of the IPSP. However, when the impulse is permitted to invade the soma-dendritic membrane, there is a large increase in the size of the IPSP. It can be concluded, then, that the observed increment in the IPSP is attributable to a synaptic pathway involving the soma-dendritic membrane.

Our results directly demonstrate the presynaptic action of dendrites, for both mitral and granule cells of the olfactory bulb, and show that the reciprocal pathway remains intact when all sodium-dependent propagated action potentials are blocked by TTX. Such a pathway provides for an all-or-none self-inhibition of mitral cells (*12*). These cells are also subjected to lateral inhibition, as is apparent from observations that orthodromic and antidromic stimuli below threshold for generating action potentials in the impaled cell nevertheless evoke small IPSP's.

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20 August 1979; revised 19 December 1979

Role of Nitrogen Dioxide in the Biosynthesis of Nitrosamines in Mice

Abstract. *Groups of three to four mice were gavaged with aqueous solutions of 2 milligrams of morpholine, after which they were exposed to nitrogen dioxide in inhalation chambers at concentrations of 0.2 to 50 parts per million for up to 4 hours. At sequential intervals during the exposure, mice were frozen and pulverized in liquid nitrogen, and the mice powder was extracted with ice-cold 35 percent aqueous methanol and dichloromethane; organic-phase concentrates were analyzed for* N-*nitrosomorpholine with a thermal energy analyzer interfaced to a gas chromatograph. The* N-*nitrosomorpholine yields, ranging up to about 2.3 micrograms per mouse, were time-dependent relative to the duration of exposure to nitrogen dioxide and dose-dependent relative to the concentrations of nitrogen dioxide; control levels (in mice that were gavaged with morpholine or distilled water and then exposed to air instead of nitrogen dioxide) were less than 5 nanograms per mouse. These preliminary studies demonstrate the in vivo nitrosating potential of nitrogen oxides.*

Concern about the public health hazards of carcinogenic nitrosamines in air, water, food, and consumer products is growing (*1*). This is paralleled by concern over the wide environmental distribution of nitrosamine precursors, nitrite and amines (*2*), from which nitrosamines can be readily synthesized both in vitro and in vivo (*3*, *4*). The role of nitrite as a nitrosating agent is well documented; there is more limited evidence on nitrosation by nitrogen oxides (NO_x) of secondary amines in the liquid or solid phase (*5*), in the gas phase (*6*), and in vitro in plasma (*5*) or lung homogenates (*7*). To our knowledge, there are, however, no available data on in vivo nitrosation by NO_x. Nitrogen oxides are common atmospheric pollutants, found in a wide range of anthropogenic sources, largely resulting from the combustion of fossil fuels, including auto exhaust, emissions from stationary sources such as utility plants, and a wide range of high-temperature combustion processes such as welding and foundry work (*8*). Addition-

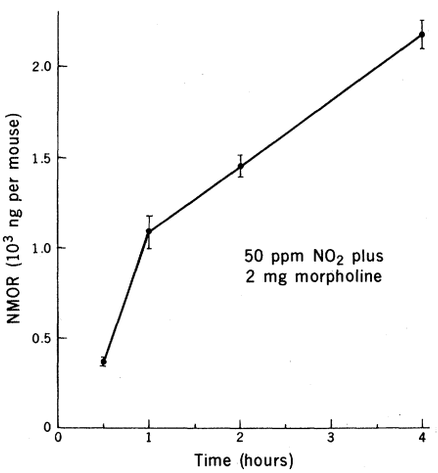

Fig. 1. Time course of NMOR biosynthesis in mice. Groups of three to four male ICR mice were gavaged with freshly prepared solutions of 2 mg of MOR (Aldrich Chemical) in 0.2 ml of distilled water and immediately placed in exposure chambers (Nalge desiccators, modified for gas inflow from the bottom and exhaust from the top). Mice were then exposed to 50 ppm NO_2 (three to four mice per chamber, 5 cubic feet per hour, 20 volume changes per hour) at intervals of from 0.5 to 4 hours. The required concentrations of NO_2 were produced by mixing stock NO_2 (custom grade, Union Carbide) with air at an appropriate flow rate, prior to introduction into the chambers; we checked the accuracy of the exposure mixtures by periodically monitoring and analyzing the NO_2 in the exhaust from the chambers, using the Griess-Saltzman reaction (*19*). Concurrent controls consisted of two mice exposed in separate chambers to NO_2 alone for 4 hours, additional controls were gavaged with 2 mg of MOR or 0.2 ml of distilled water and exposed to air for identical periods in separate chambers. After exposure to NO_2, the mice were killed by freezing in liquid nitrogen and blended to a fine powder (*20*). Two or three aliquots (approximately 8 g each) were taken from each mouse powder and blended with 75 ml of ice-cold 35 percent aqueous methanol in a Waring Blendor (5 minutes, medium speed); a known amount of a nitrosamine standard [152 ng of di-*n*-propylnitrosamine (DPN), Aldrich] was then added, and blending continued for 1 to 2 minutes. Homogenates were divided in half and centrifuged (5000*g*, 25 minutes, 5°C; swinging bucket), supernatant was removed, and the pellets were extracted again with cold 35 percent methanol. The pooled supernates were extracted (twice) with an equal volume (total, 150 ml) of dichloromethane [(DCM), Burdick and Jackson] (*21*), and the organic layer was dried by passage through a cotton gauze (Ex-tube, Analytichem International) and concentrated to 2 ml in a Kuderna Danish concentrator (Kontes, 250 ml) kept in a 65°C bath. Aliquots (20 μl) of the concentrates from each of two or three powder samples were injected into the thermal energy analyzer–gas chromatograph (Thermo Electron modified model TEA-502) (*22*) for NMOR analysis. Peaks were identified and quantitated by comparison with the retention time and response of reference nitrosamines (*23*). The plotted values are corrected for any background control NMOR levels and for the DPN standard recoveries and represent means of three to four mice ± the standard deviation.

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al sources of NO_x include mainstream and sidestream tobacco smoke (*9*) and domestic gas stoves (*10*).

We present here results of preliminary studies demonstrating in vivo nitrosation in mice of an exogenous amine, morpholine (MOR) (*11*), by inhaled NO_2. We also present data on the time- and dose-response relationships of the resulting *N*-nitrosomorpholine (NMOR) biosynthesis.

The time dependence of NMOR biosynthesis is illustrated in Fig. 1. When mice gavaged with 2 mg of MOR were then exposed to 50 parts per million (ppm) of NO_2 for 0.5 hour, the NMOR yields were 370 ± 12.5 ng per mouse (or 0.02 percent of the MOR administered). The NMOR yields increased about three times when the NO_2 exposure time increased from 0.5 to 1 hour, and then increased linearly as the NO_2 exposure time increased to 4 hours, reaching 2230 ± 138.6 ng per mouse (or 0.11 percent of the MOR administered). Variability within an experimental group (three to four mice) ranged from ± 3.3 to ± 13.3 percent, the higher degree of variability corresponding to the longer exposure periods. The NMOR yields in controls that were gavaged with MOR and then exposed to air instead of NO_2 were less than 5 ng per mouse; the NMOR yields were undetectable in controls that were either exposed to NO_2 alone or given only distilled water. The dose dependence of NMOR biosynthesis as a function of NO_2 exposure levels is presented in Fig. 2. Yields of NMOR in MOR-treated mice increased with NO_2 concentrations from 0.2 to 50 ppm. The exposure of MOR-treated mice to as low as 0.2 ppm of NO_2 for 4 hours resulted in a NMOR biosynthesis of 56 ± 6 ng per mouse, significantly higher than the values for MOR and NO_2 controls ($P < .001$); comparable yields of NMOR were produced by exposure to 0.2 ppm of NO_2 for 16 hours, but the yields were less than one-half (21.6 ± 1.6 ng per mouse) as a result of exposure to 0.2 ppm of NO_2 for 0.5 hour (*12*).

Experimental controls for artifactual formation of NMOR during analysis showed the following: (i) The addition of 50 to 250 mg of sodium ascorbate to the powder derived from mice that had been gavaged with 2 mg of MOR and exposed to 50 ppm of NO_2 for 4 hours (which was then homogenized and extracted as described in Fig. 1) resulted in a slight (less than 5 percent) but insignificant decrease in the NMOR yields as compared to approximately 2230 ng of NMOR per mouse in controls without ascorbate. (ii) When mice were exposed to 50 ppm of NO_2 for 4 hours, then gavaged with 2 mg of MOR, and frozen immediately for homogenization and extraction, less than 200 ng of NMOR per mouse were detected (that is, less than 10 percent of the yields in controls gavaged with MOR and then exposed to NO_2 for 4 hours). (iii) Similar yields were found when 2 mg of MOR was added to the powder derived from mice that had been exposed to 50 ppm of NO_2 for 4 hours, prior to homogenization and extraction.

Our data demonstrate the biosynthesis of NMOR in mice after in vivo nitrosation of MOR by NO_2. The NMOR yields in these experiments are likely to reflect a wide range of factors, including the competing effects of MOR clearance and NMOR catabolism and excretion (*13*) and macromolecular binding of NMOR (*14*). Artifactual formation of NMOR during analysis could account for as much as 5 to 10 percent of the observed yields.

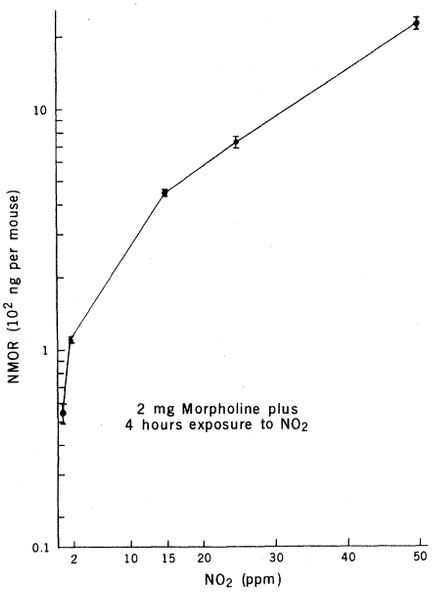

Fig. 2. The NO_2 concentration dependence of NMOR biosynthesis in mice. The procedures were the same as in Fig. 1, except that mice, gavaged with 2 mg of MOR, were subsequently exposed to 0.2 to 50 ppm of NO_2 for 4 hours, Dynacal permeation tubes (Metronics) were used to generate 0.2 and 15 ppm of NO_2 in the exposure chambers. For generating 0.2 ppm of NO_2, air (2.5 liter/min) was introduced into a plastic tube (15 cm long, 1.5 cm inside diameter) containing a metal wafer device (5.72 cm long, 1.43 cm in diameter) kept in a 35°C water bath; the other end of the plastic tube was connected to the exposure chamber. The 15 ppm of NO_2 was generated in a similar manner, except that a standard emission tube (10 cm long), kept at 42°C, was used. Both the wafer device and standard emission tubes were conditioned overnight prior to use, and NO_2 concentrations were colorimetrically confirmed (*19*). Exhaust from the exposure chambers was passively eliminated in a chemical hood.

These data do not, however, identify the site or mechanisms of the NMOR biosynthesis. Liquid-phase nitrosation of amines by NO_x is believed to be mediated by the reactive tautomeric forms of N_2O_3 or N_2O_4, or by a free radical process (*5*). Nitrosation of MOR in vivo could occur in the pulmonary capillaries or in the systemic blood stream by the nitrosating radical formed after absorption of NO_2; NO_x from sources such as tobacco smoke are known to be readily absorbed into the blood during inhalation (*8, 9*). Nitrous acid, formed as a result of NO_2 inhalation, could be secreted into saliva and then react in the stomach with MOR.

The possibility of in vivo nitrosation of amines by NO_x has clear public health implications (*2, 8, 9, 15*). Potentially available for in vivo nitrosation are the endogenous amines [such as dimethylamine (DMA)] at naturally occurring levels, endogenous amines (such as DMA) from exogenous sources such as meat or fish, and exogenous amines (such as MOR) from sources such as air pollutants. Recent findings of high concentrations of NMOR in rubber-curing areas in tire factories (a high incidence of lung cancer has been observed in persons engaged in this work) and high concentrations of dimethylnitrosamine in leather tanneries have not been investigated epidemiologically (*16*). Limited epidemiological studies have suggested some relationship between the ambient NO_x concentrations and an urban excess of cancer (*17*), although further investigations are clearly needed. Speculations have also been voiced about the possibility of an NO_x-nitrosamine-cancer link (*18*). Our studies demonstrate the possible hazards due to NO_x-mediated nitrosation of endogenous and exogenous amines.

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21. Pesticide-grade methanol (Fisher Chemical) and glass-distilled DCM were used. Concentrated samples of DCM and methanol, DCM extracts of distilled water, and aqueous solutions of MOR were free of NMOR and other nitrosamines when checked with the thermal energy analyzer–gas chromatograph; detection limit, 0.1 to 0.5 part per billion. We synthesized the NMOR in our laboratory by reacting sodium nitrite with MOR solution in H_2SO_4 [B. Prager and P. Jacobson, Eds., *Beilstein's Handbuch der organischen Chemie* (Springer, Berlin, ed. 4, 1937), vol. 27, system 4190, p. 8.
22. A stainless steel column (4.6 m by 3.2 cm) packed with 10 percent Carbowax 20M plus 0.5 percent KOH on Chromosorb WHP 80/100 was used under isothermal conditions at 175°C; argon carrier gas, used at an inlet pressure of 60 pounds per square inch (4.1 atm) achieved a flow rate of 22 ml/min.
23. Recovery of the DPN internal standard ranged from 85 to 90 percent, all NMOR values were corrected for the DPN recovery. The NMOR and DPN values from 8- to 16-g aliquots of powder, varied less than 5 percent from the average, on a per-gram weight basis. Low concentrations of DMN (2 to 3 ng per mouse) were occasionally found in untreated controls or controls exposed to NO_2 or MOR, or both.
24. This work was supported by U.S. Environmental Protection Agency grant R-805431. The assistance of R. Aslan, D. Mataitis, and M. Johnson is gratefully acknowledged.

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Intracellularly Injected Cobaltous Ions Accumulate at Synaptic Densities

Abstract. *Physiologically identified neurons in the locust were iontophoretically injected with a mixture of cobaltous and potassium ions. After being fixed for electron microscopy, 2.5-micrometer sections of the epoxy-embedded ganglia were intensified with silver. The intensified material was resectioned and examined in the electron microscope. The cobalt-silver precipitate appeared as discrete densities. Localized accumulations of the precipitate were seen within the injected cell along the neuronal membranes and especially at synapses. Location and recognition of the stained neuron in the electron microscope was facilitated by the tendency of the cobaltous ions to aggregate at the synaptic sites.*

The need for an approach combining anatomy with physiology in the study of neuronal circuitry has been recognized for more than a decade (*1*). The introduction of dyes that could also be used as recording solutions in microelectrodes (*2, 3*) has made such combined studies feasible. Although a variety of dyes can provide excellent results for light microscopy, all of the previously reported techniques have had limited usefulness for the electron microscope (EM). Earlier methods with cobalt resulted in poor fixation, thought to be caused by the high concentration of cobaltous ions required for them to be seen in the EM (*3–5*). Procion dyes also disrupt organelles and may trigger phagocytosis by surrounding glia (*6, 7*). The diffuse, electron-opaque horseradish peroxidase reaction product obscures the ultrastructure of the injected cell and greatly increases electrode resistance, making recording more difficult (*7, 8*). Here I report a technique for silver intensification of intracellularly injected cobaltous ions which overcomes these problems.

Neurons were physiologically identified and filled by cobalt-potassium-filled electrodes (*9*), which have both good recording and good staining characteristics. This technique allows unambiguous identification of the stained neuron, including its synaptic sites, without the necessity of EM serial reconstruction. The cobalt-silver precipitate is discrete and highly electron-opaque making it easily seen in the EM. The results presented here are from three fast extensor tibiae motoneurons stained in one mesothoracic and two metathoracic ganglia of the locust *Schistocerca americana gregaria*. Successful results with this technique have also been obtained for 15 other neurons in the locust, including local nonspiking interneurons (*10*). A preliminary report of the technique has been made elsewhere (*11*).

Neurons were identified in the meso- and metathoracic ganglia by standard techniques (*12*). An isotonic saline was used (*13*). After physiological identification of the motoneuron, cobaltous ions were injected into the soma with 10- to 20-nA current pulses of 50-msec duration and 10-Hz frequency for 40 minutes. The ganglion was then excised, transferred to a Vaseline well on a glass slide, and flooded with fixative (2.5 percent glutaraldehyde, 0.05*M* sodium phosphate buffer, 0.2*M* sucrose, *p*H 7.2). After 15 minutes, the extreme edge of the ganglion opposite to the soma of the filled neuron was cut away to ensure that the solution would penetrate. The solution in the well was changed to 0.5 to 1.0 percent ammonium sulfide in buffer (0.05*M* sodium

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