Biological Roles of Nitric Oxide

This previously elusive and obscure chemical is proving to be of vital physiological significance. Nitric oxide may be the first of a novel class of neurotransmitters

by Solomon H. Snyder and David S. Bredt

Small, simple and highly toxic, nitric oxide seems an unlikely biological jack-of-all-trades. Indeed, most of the body's functions are regulated by extraordinarily large and complex proteins and compounds. The tools of modern molecular biology have revealed such elaborate chemicals as the hormone testosterone and the immune system protein gamma interferon.

Such chemical complexity seems almost ostentatious when compared with seemingly plain and unassuming nitric oxide. Nitric oxide, or NO, is a gas under atmospheric conditions. It is not to be confused with nitrous oxide, or N_2O, the laughing gas used as an anesthetic. Nitric oxide is notoriously noxious because of its free-radical structure: it possesses an extra electron, making it highly chemically reactive. And although it has long been known that bacteria contain nitric oxide, no one anticipated that such a reactive agent would have a crucial function in mammals.

Five years ago this belief was dispelled when a series of discoveries from many different avenues of research came together, revealing the major biological roles of nitric oxide. Studies have shown that the chemical is perhaps one of the most important messenger molecules. It enables white blood cells to kill tumor cells and bacteria, and it allows neurotransmitters to dilate blood vessels.

Nitric oxide simultaneously serves as a messenger for neurons, much like a neurotransmitter, in the brain and other parts of the body. In fact, nitric oxide may prove to be the first in a series of neurotransmitters unlike any of those previously elucidated. Understanding the molecular mechanisms of this potent compound, its distribution and its relation to other important bodily agents has led to clues that may be illuminating for memory research and for the treatment of certain neurodegenerative disorders.

Early studies of nitric oxide suggested the compound was anything but beneficial. Nitric oxide is extremely labile, that is, short-lived. It exists for about six to 10 seconds and then is converted by oxygen and water into nitrates and nitrites. Although humans excrete nitrates, scientists used to think these compounds derived solely from dietary sources. Therefore, in 1956, when P. N. Magee and J. M. Barnes of the Medical Research Council Laboratories in Surrey, England, reported that the body converts nitrates from cured foods to carcinogenic nitrosamines, people rushed to change their eating habits. Bacon and other cured foods high in nitrates were shunned.

In 1981 Steven R. Tannenbaum and his associates at the Massachusetts Institute of Technology noted that humans and rats fed low-nitrate diets still excreted substantial amounts of nitrates. Obviously, diet was not the sole source. Where did the compounds originate? Tannenbaum found a valuable clue in one of his subjects: a man who excreted very high levels of urinary nitrates while he had infectious diarrhea. Inflammatory processes associated with the diarrhea were apparently responsible for nitrate formation. Tannenbaum noted that injections of bacterial endotoxin, which causes an inflammatory response to bacteria, stimulated nitrate excretion in rats.

The precise source of nitrate formation and its relation to inflammatory responses were ultimately pinned down by Michael A. Marletta of the University of Michigan and his student Dennis J. Stuehr and by John B. Hibbs, Jr., of the University of Utah. Marletta had been a student of Tannenbaum's at M.I.T., and he remained intrigued about the part the immune system played in the endotoxin-induced nitrate formation. Marletta found that mice with a certain genetically determined macrophage deficiency excreted few nitrates. He thereby established an association between the presence of macrophages and the presence of nitrates.

Marletta probed further. He isolated cultures of the missing macrophages. He then introduced endotoxin into the culture along with gamma interferon, an immune modulator protein that activates other immune cells and that is formed by *T* lymphocytes. After this infusion the macrophages were suddenly able to produce nitrates.

By selectively testing different aspects of the cultures, Marletta also discovered macrophages could not produce nitrates when the amino acid arginine—normally present in the incubation medium—was absent. That finding enabled him to prove that a specific enzyme in the macrophages converts arginine into an intermediate chemical. The chemical turned out to be nitric oxide, which is quickly transformed into nitrites and nitrates.

Meanwhile Hibbs, working independently, was evaluating the ability of

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STAINED NEURONS become rust colored if they contain nitric oxide synthase (NOS), the enzyme that converts arginine to nitric oxide. Grayish blue-stained neurons do not contain NOS.

macrophages to kill tumor cells and bacteria. He cultured tumor cells with macrophages and noted that the tumor-killing ability of the macrophages disappeared when arginine was removed from the medium. Hibbs also proved arginine was converted not only into nitrates but also into the amino acid citrulline. In this way, he provided evidence that a specific enzyme produced nitric oxide from arginine.

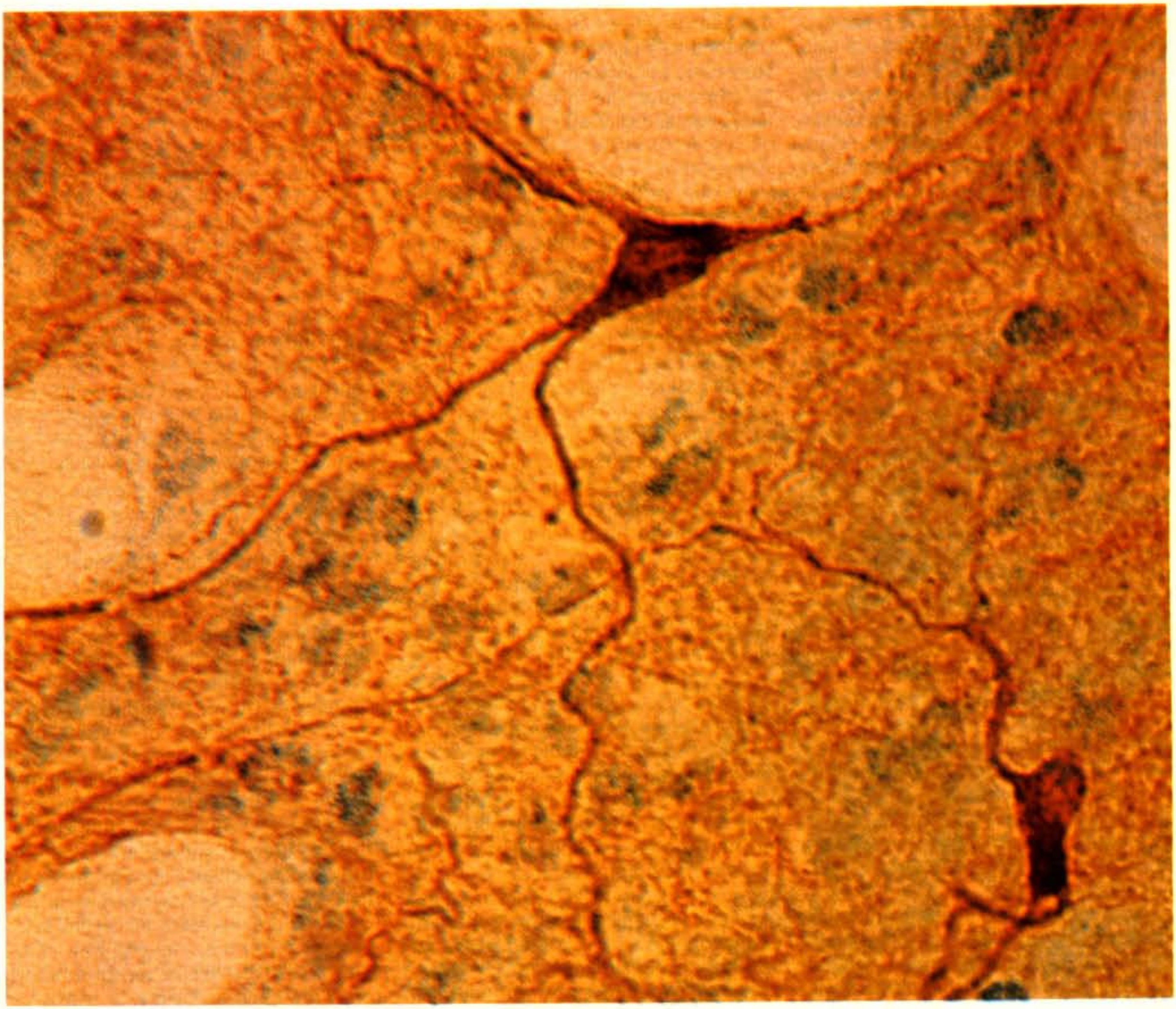
Hibbs went on to demonstrate that nitric oxide gas was as toxic to the tumor cells as were the activated macrophages. And he identified the first inhibitor of the enzyme synthesizing nitric oxide. Hibbs did this by showing that a derivative of arginine—specifically a methyl derivative—blocked both the formation of nitrates and the macrophage's tumor-destroying prowess. Without an enzyme producing nitric oxide from arginine, no macrophage defense could be mounted.

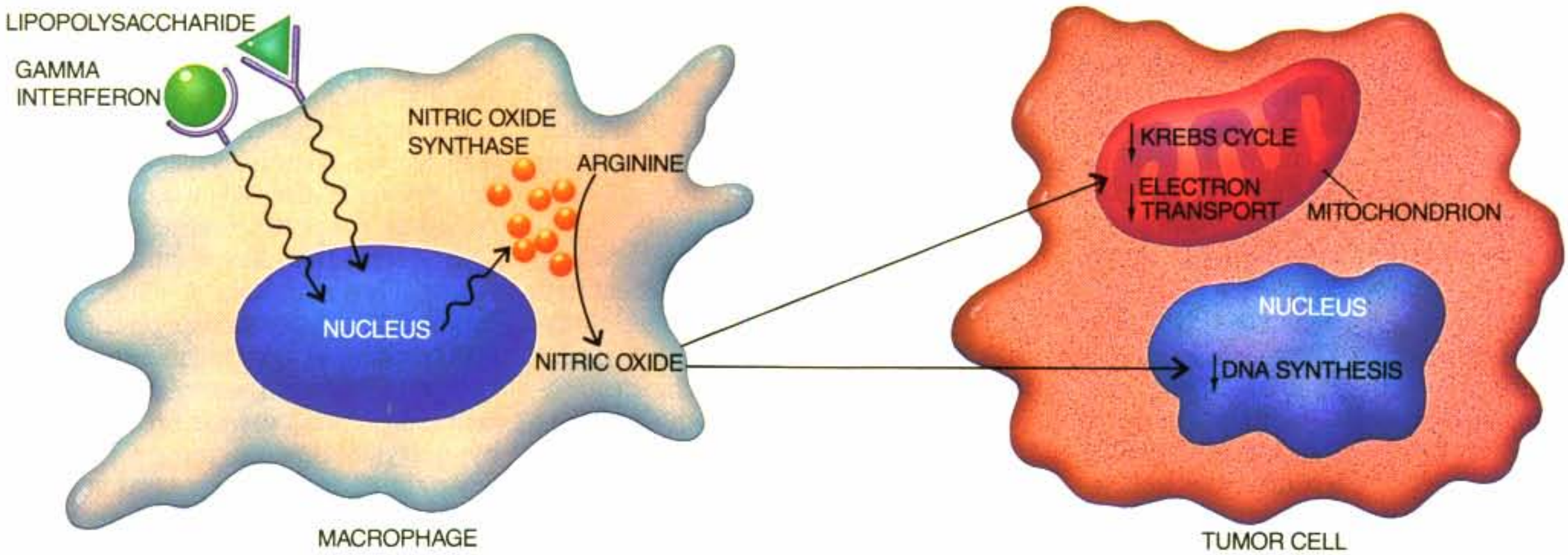
This is where research stood several years ago. When macrophages are activated by endotoxins or *T* cells, they respond by converting arginine into nitric oxide. The toxic, free-radical nitric oxide, in turn, allows macrophages to kill bacteria, fungi and tumor cells.

In a completely unrelated series of investigations, researchers identified nitric oxide as a messenger molecule. There are two parts to this aspect of the nitric oxide story. The first entails the mechanisms by which neurotransmitters dilate blood vessels; the second concerns the drugs that relieve the symptoms of angina, a form of heart disease in which the coronary arteries of the heart constrict. Both lines of research coincided recently to reveal more about the intricacies of nitric oxide's functions.

Blood vessels are dilated by neurotransmitters that cause the muscle layer of the vessels to relax, such as acetylcholine. Counterbalancing this effect are other neurotransmitters that contract the muscle and constrict blood vessels, such as norepinephrine. Because norepinephrine receptors occur directly on muscle cells, most scientists assumed the cells would also bear receptors for acetylcholine.

In 1980 this assumption proved wrong. That year, Robert F. Furchgott, a prominent cardiovascular pharmacologist working at the Downstate Medical Center in Brooklyn, noticed that the relaxation of blood vessels brought about by acetylcholine no longer occurred when the endothelial layer was stripped from the vessels. Endothelium is the thin layer of cells on the interior surface of blood vessels, immediately adjacent to the muscle layer. In a series of experiments, Furchgott demonstrated that acetylcholine acts on receptors located on the endothelial cells. This action provokes the release of a small molecule that diffuses to the adjacent muscle layer and relaxes it.





IMMUNE SYSTEM STIMULI gamma interferon and lipopolysaccharide transmit signals to a macrophage nucleus. The signals cause production of nitric oxide synthase, the enzyme that converts arginine to nitric oxide (NO). NO destroys tumor cells by inhibiting the energy-producing Krebs cycle and electron transport activities as well as DNA synthesis.

The mysterious molecule, or endothelium-derived relaxing factor (EDRF), as it was soon called, was nearly impossible to identify. Numerous investigators, including Furchgott and Louis J. Ignarro of the University of California at Los Angeles, tried unsuccessfully to isolate the labile compound. Despite their inability to identify EDRF, the researchers made a significant discovery. They proved EDRF stimulates the formation of cyclic guanosine monophosphate (GMP), a so-called second messenger for neurotransmitters and hormones. Cyclic GMP is related to the better-known second-messenger molecule, cyclic adenosine monophosphate (AMP).

Meanwhile another vein of research was being pursued, one that would come to bear on the work of Furchgott and Ignarro. Investigators, such as Ferid Murad of Abbott Laboratories, were seeking to understand the intricacies of nitroglycerin's effectiveness as a treatment for heart attack. This potent drug alleviates the symptoms of cardiac arrest by dilating coronary arteries and the veins that supply blood to the heart. Nitroglycerin, the active chemical in dynamite, was invented by Alfred Nobel, who endowed the Nobel Prize. Its therapeutic effects were well known in the late 1800s. Nobel, who suffered from angina, wrote to a friend about them: "It sounds like the irony of fate that I should be ordered by my doctor to take nitroglycerin internally." The therapeutic success of the drug resulted in numerous derivatives (the organic nitrates), which remain the mainstays of anginal treatment.

Although Nobel had discovered the compound nearly a century before, not until the late 1970s was any insight into the molecular mechanisms of nitroglycerin available. Murad, then at Stanford University, found that nitroglycerin and the organic nitrates are themselves inactive, although they elicit blood vessel relaxation once they are metabolically converted to nitric oxide. Moreover, nitric oxide relaxes muscle by stimulating the formation of cyclic GMP, just as EDRF does. The two lines of research, EDRF and nitroglycerin, seemed to have converged.

By 1986 both Furchgott and Ignarro had predicted that nitric oxide or some closely related derivative might account for EDRF's activity. Finally, in 1987, proof that EDRF is identical to nitric oxide was provided. Salvador Moncada and his associates at the Wellcome Research Laboratories in Beckenham, England, stimulated the release of EDRF from endothelial cells and monitored its relaxing effect on smooth muscle. At the same time, they chemically measured the amount of nitric oxide released from the endothelium. The endothelium released enough nitric oxide to account fully for the relaxation of adjacent muscle cells; therefore, nitric oxide is EDRF. Ignarro's group soon obtained similar results. In addition to relaxing blood vessels, nitric oxide inhibits blood clotting by preventing the aggregation of platelets. It has also been found to be the normal regulator of penile erection.

Today nitric oxide's role in the vascular system has been shown to be even more extensive. Although other substances, such as angiotensin and norepinephrine, were assumed to be the major determinants of blood pressure, nitric oxide apparently is the principal regulator of blood pressure. Several investigators have administered inhibitors of the enzyme that makes nitric oxide—nitric oxide synthase—to both animals and humans. Such treatment provokes a rapid increase in blood pressure, an increase more notable than the alterations produced by drugs influencing norepinephrine or angiotensin. Changes in the regulation of nitric oxide could be associated with hypertension or other blood pressure abnormalities.

The pace of discovery about nitric oxide verges on dizzying. Knowledge about its importance to the immune and vascular systems is itself relatively recent, but there is even fresher news: nitric oxide's function in the brain. The first hint that nitric oxide was involved in the nervous system came only in 1982. Takeo Deguchi of the Tokyo Metropolitan Institute for Neurosciences noticed that cyclic GMP formation in the brain requires arginine. Of course, at that time, no one knew that nitric oxide was a messenger molecule or that it was formed from arginine. In 1989 Moncada, at Wellcome, reasoned that arginine's role in cyclic GMP formation in the brain must relate to nitric oxide formation. In fact, he found nitric oxide–forming activity in brain tissue preparations.

Simultaneously, another body of evidence was accumulating. John Garthwaite of the University of Liverpool observed the formation of a short-lived substance that had the properties of nitric oxide when he stimulated brain tissue by administering the amino acid glutamate. Glutamate, an excitatory neurotransmitter, accounts for synaptic transmission at more sites in the brain than does any other neurotransmitter. Its effects are mediated by several subtypes of receptors. The one best characterized is the NMDA receptor, short for *N*-methyl-D-aspartate, a synthetic

amino acid that acts selectively at this subtype of glutamate receptor. At NMDA receptors, glutamate opens calcium ion channels, gatekeepers of neuronal transmission, thereby sending a strong excitatory impulse.

When released in large amounts, however, glutamate can cause damage by opening these channels. For example, the death of neurons during most strokes results perhaps from a cascade of glutamate acting on cells already deprived of oxygen. The added stress of having to fire more rapidly because of the glutamate stimulation exhausts and then kills the cells [see "Stroke Therapy," by Justin A. Zivin and Dennis W. Choi; SCIENTIFIC AMERICAN, July 1991]. Neuronal damage in animals in which strokes have been induced can for the most part be prevented by drugs that block NMDA receptors. The private sector has been quick to respond. NMDA antagonists are being developed by several companies as potential treatments for stroke.

Garthwaite's observation that stimulating NMDA receptors releases nitric oxide implicated the agent as a glutamate mediator. So in early 1989 we decided to investigate a possible role of nitric oxide in synaptic function. Because nitric oxide was known to act through cyclic GMP in blood vessels, we looked for a part of the brain in which glutamate had been shown to influence cyclic GMP. Fortunately, in the 1970s James Ferrendelli of Washington University had added glutamate to slices from the cerebellum and observed a rapid, pronounced increase in cyclic GMP.

Using similar cerebellar brain preparations, we developed a technique to measure the activity of the nitric oxide–forming enzyme, nitric oxide synthase. Because arginine produces nitric oxide and citrulline in equal proportions, we monitored the conversion of radioactive arginine to citrulline. Whatever the amount of citrulline we measured, it would therefore indicate that the same amount of nitric oxide had been produced. By this method we found that nitric oxide synthase activity tripled when we added NMDA or glutamate to the slices. We were struck by the extreme rapidity of this effect: it took place in a matter of seconds. This discovery was somewhat perplexing because increasing the activity of an enzyme usually requires a long time.

In the same brain slices, we confirmed that NMDA provoked large increases in cyclic GMP levels. We decided to examine whether there was a causal link between the formation of nitric oxide and of cyclic GMP. This question was easily addressed by adding inhibitors of nitric oxide synthase—specifically the methyl arginine derivative mentioned above—to the slices. Methyl arginine blocked the formation of cyclic GMP at the same concentrations as it inhibited nitric oxide synthase. Because both processes were inhibited by the same amount of methyl arginine, it seemed clear the two were related. Garthwaite and Moncada also observed this blockade of cyclic GMP formation.

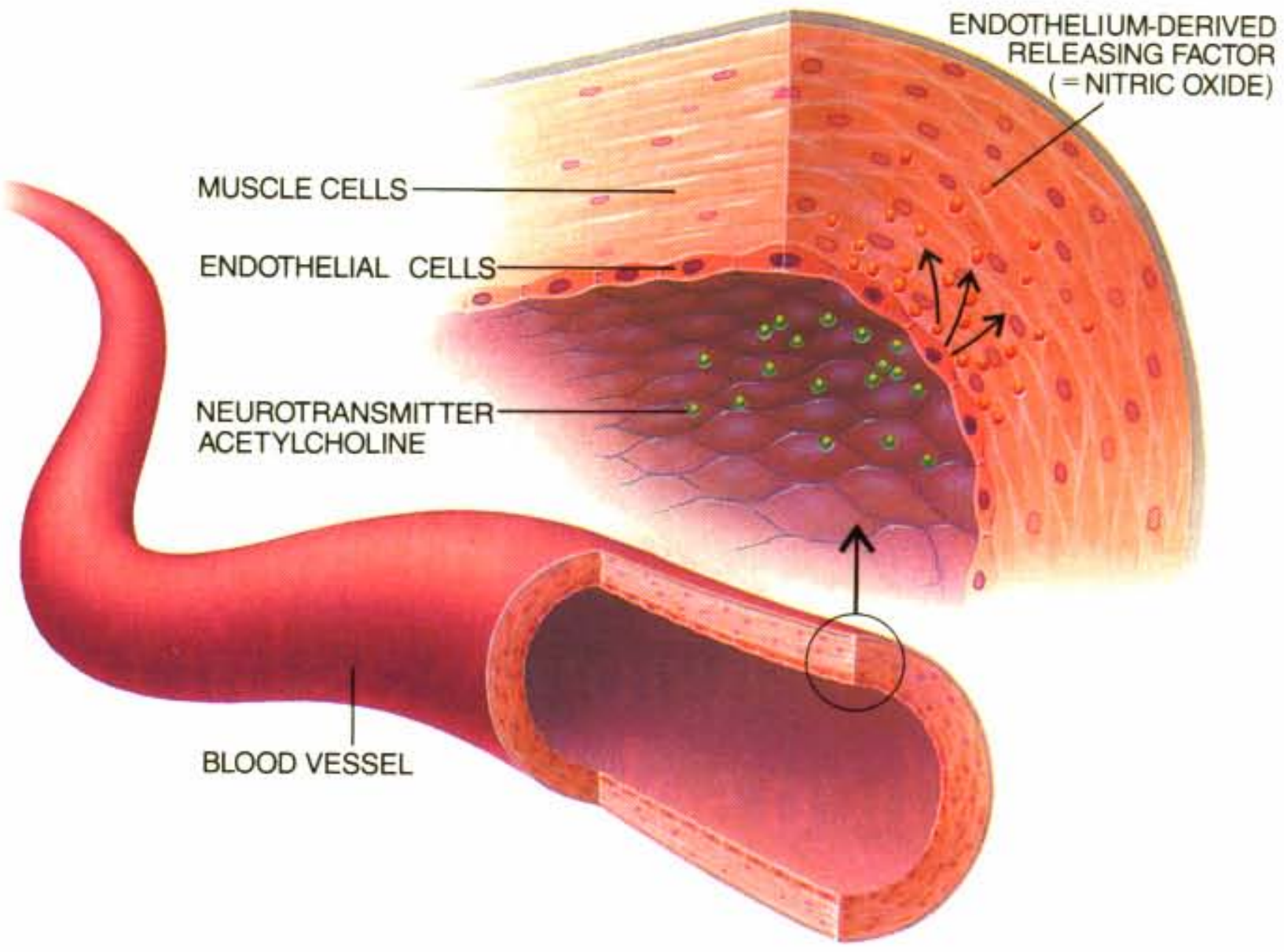
Our next step was to explore where nitric oxide worked in the brain. Normally, neuroscientists glean such functions of an important molecule by finding its specific neuronal locations, which are, in turn, associated with particular biological pathways and functions. But trying to locate a short-lived molecule such as nitric oxide seemed hopeless. Instead we attempted to localize nitric oxide synthase.

One of the most efficient means of localizing proteins is provided by immunohistochemistry. An antibody to the molecule that is to be traced is applied to tissue samples. It binds with the molecule, or antigen, in question. Various techniques, including staining, are then used to mark where the antibody is bound to its antigen.

Before we could proceed with our immunohistochemical experiments, however, we had to obtain antibodies to nitric oxide synthase. And before we could do that, we had to isolate the enzyme itself. This was no easy task. We tried numerous purification techniques, but with each one we quickly lost all enzyme activity. We therefore reasoned that some aspect of the purification procedure was removing a crucial chemical that served as a cofactor, or assistant, in the enzyme's activity. So we considered trying to purify this hypothetical cofactor.

But that approach seemed rather complicated, and we turned instead to guesswork. Moncada had discovered that the synthesis of nitric oxide required the presence of calcium. Calcium often acts by binding to a ubiquitous cofactor called calmodulin. We added a small amount of calmodulin to some of our enzyme preparations and immediately saw a profound enhancement of enzyme activity. Fortuitously, calmodulin was the missing crucial cofactor for nitric oxide synthase.

Recognition of the association between nitric oxide, calcium and calmodulin enabled us to proceed with the purification of our enzyme. More important, it explained why NMDA receptors set in motion nitric oxide synthesis so quickly after being triggered by glutamate. As is well known, glutamate causes synaptic transmission at NMDA receptors by opening the ion channels that promote the movement of calcium ions from the exterior to the interior of neurons. Therefore, glutamate causes calcium to move into cells; the calcium ions then bind to calmodulin and acti-



BLOOD VESSELS DILATE when a neurotransmitter, such as acetylcholine, binds to endothelial cells on the vessel's inner walls. These cells release endothelium-derived releasing factor (EDRF), which travels to adjacent muscle cells and causes them to relax. In 1987 EDRF was found to be identical to nitric oxide.

vate nitric oxide synthase. This entire process takes place in milliseconds.

Once we purified the nitric oxide synthase protein, we and Paul M. Hwang, a doctoral student in our laboratory, developed antibodies against it. We then tracked down its presence in the brain and the rest of the body. Our most dramatic observation was that nitric oxide synthase occurs almost entirely in neurons. Neurons make up only 15 percent of brain cells; the other 85 percent are glial cells that provide metabolic and other support for neurons. Yet no enzyme was detected in glia.

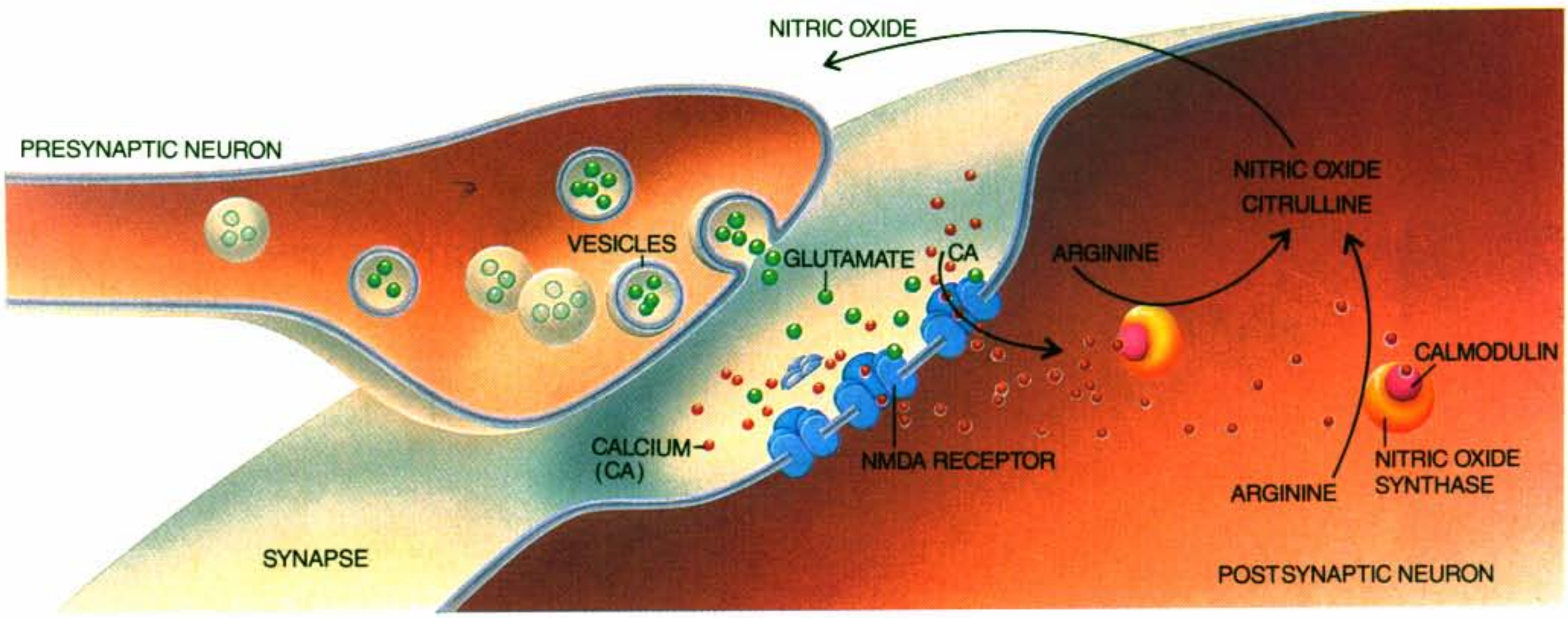
Interestingly, nitric oxide synthase was present only in discrete populations of neurons. In the pituitary gland, for instance, the enzyme exists in neurons whose cell bodies lie in the hypothalamus, whence they extend into the posterior lobe of the pituitary gland. These particular neurons synthesize and release the hormones vasopressin and oxytocin. In the adrenal gland, nitric oxide synthase is highly concentrated in a network of neurons that stimulate adrenal cells to release epinephrine, or adrenaline. In the intestine the enzyme resides in a collection of neurons referred to as the myenteric plexus. These nerve cells regulate peristalsis. In the cerebral cortex, however, the enzyme occurs in only about 2 percent of the neurons. Outside the brain, nitric oxide synthase is found in the endothelial layer of blood vessels.

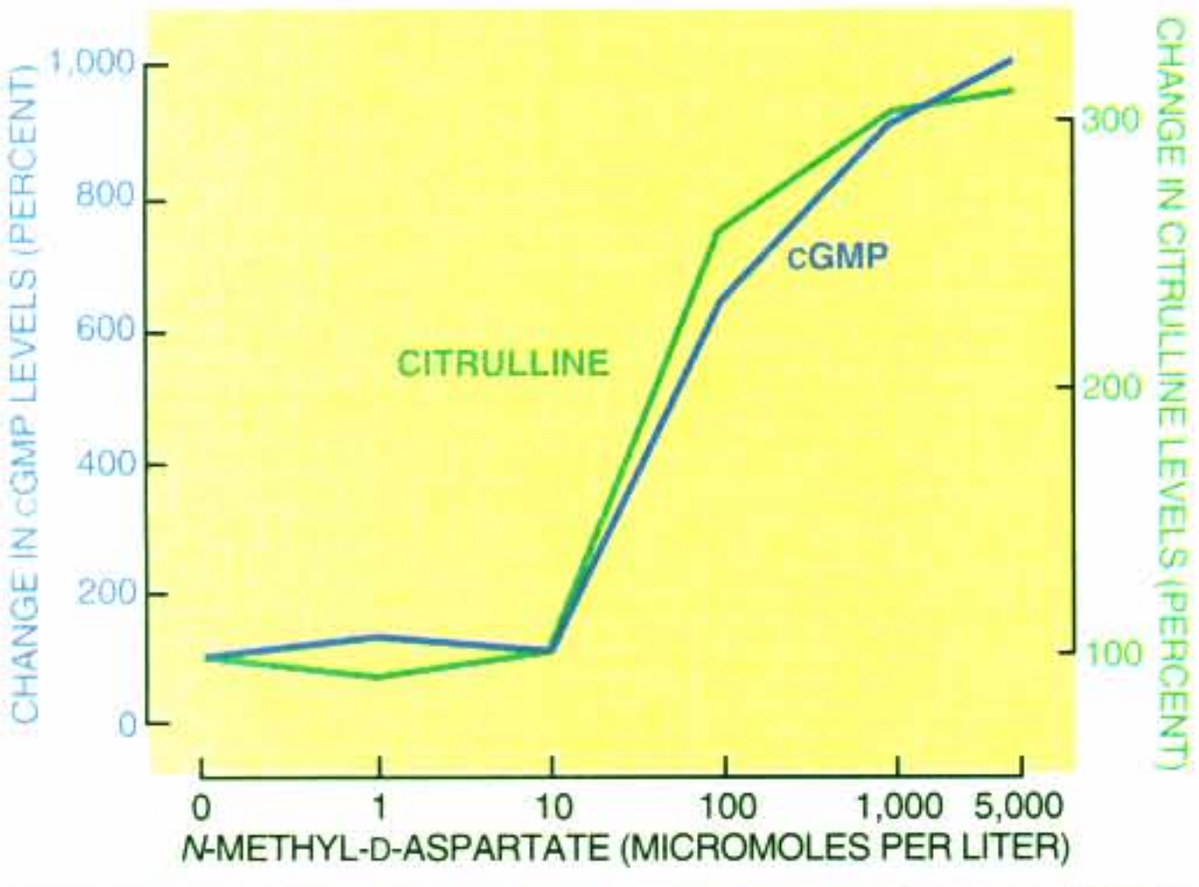
The localizations we observed are extraordinary in that nitric oxide synthase is present predominantly in neurons. The functions of nitric oxide in nerve cells must therefore be comparable in importance to its functions in macrophages and blood vessels, perhaps even more so.

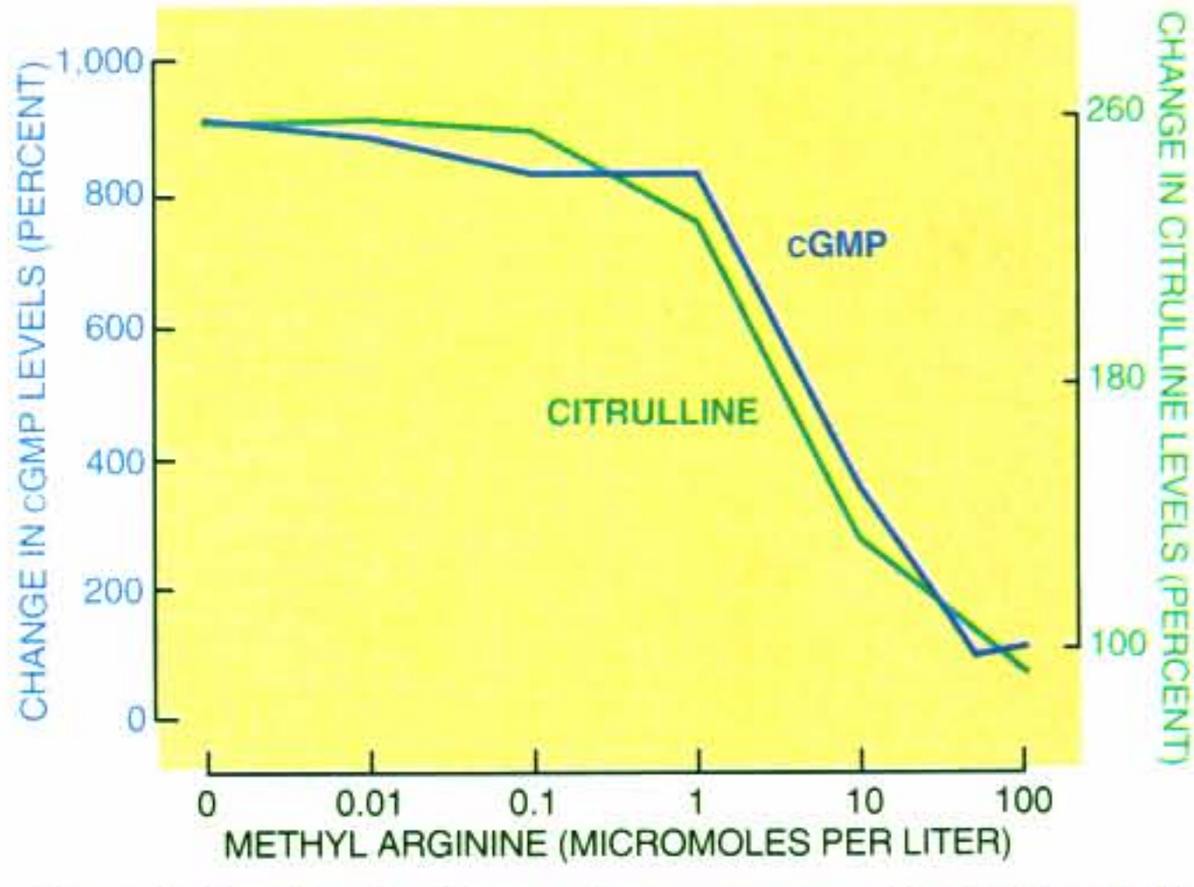
Although we successfully traced nitric oxide throughout the brain, our findings did not immediately suggest any clear function for the compound. The localizations seemed rather mysterious because they did not match the placement of any known neurotransmitter. But a breakthrough was soon made when we noticed that the peculiar localization of nitric oxide synthase in the cerebral cortex resembles another peculiar pattern of neurons, those stained by a certain dye.

This stain was developed by the British histochemist Anthony Pearse of the University of London in the mid-1960s. When Pearse stained brain slices with a dye called nitro blue tetrazolium, he observed that certain neurons turned bright blue when he added an enzyme cofactor called reduced nicotinamide adenine dinucleotide phosphate (NADPH). These neurons, called diaphorase neurons, made up about 2 percent of the cerebral cortex. NADPH donates electrons for oxidative enzymes, and so diaphorase was presumed to mediate a form of oxidation.

Few researchers, however, were ini-







NEURONAL NITRIC OXIDE is released when glutamate binds with the NMDA receptor (*top*). Binding causes calcium ions to enter the neuron; the ions bind to calmodulin, which activates NOS. NOS converts arginine to citrulline and NO. As shown below, levels of second-messenger cyclic GMP and citrulline increase when the receptor is activated (*left*). When the receptor is blocked by methyl arginine (*right*), levels of these compounds decrease, marking a decrease in NO.

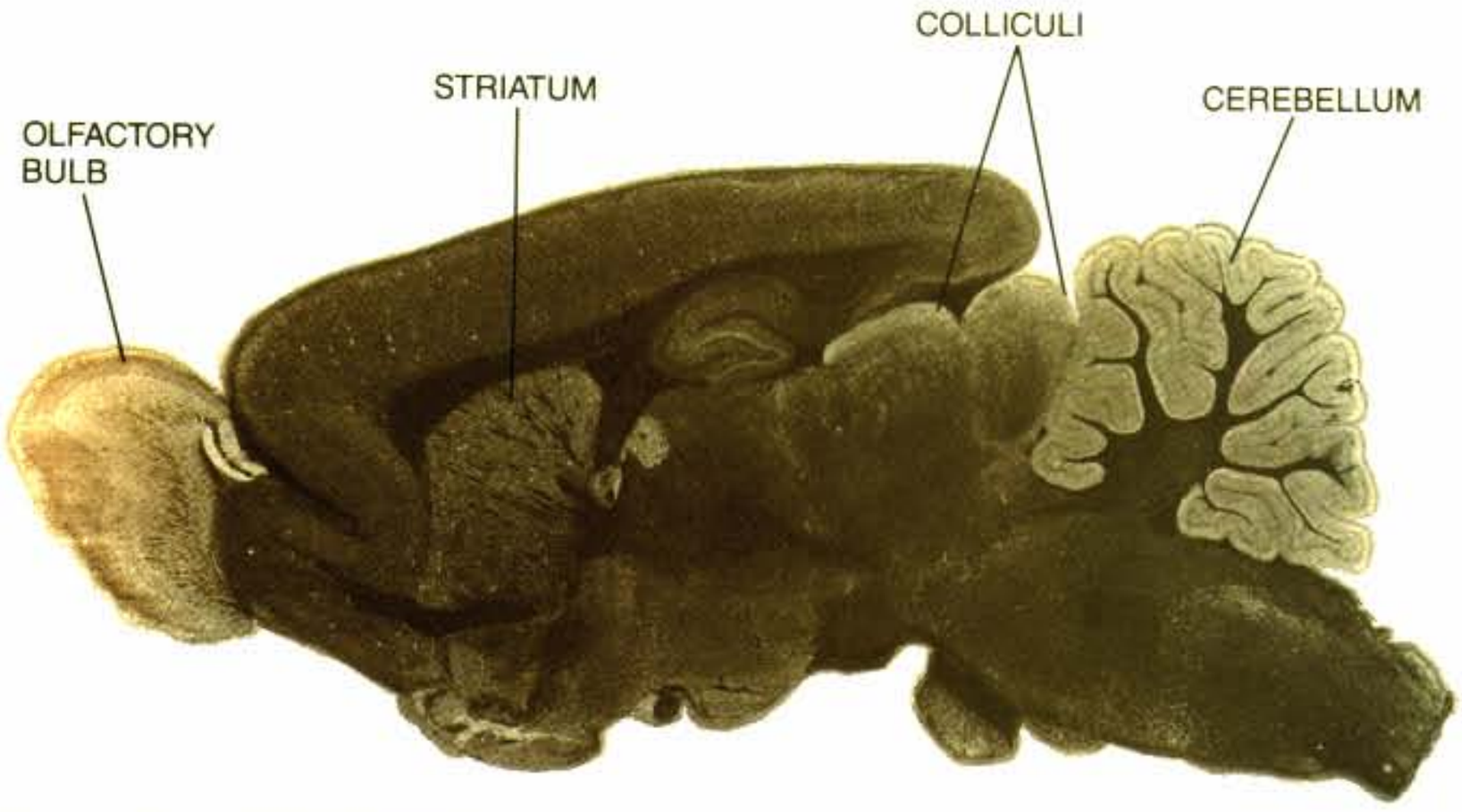
tially interested in this stain. In the mid-1980s M. Flint Beal and Joseph B. Martin of the Massachusetts General Hospital observed that neurons stained by this dye were selectively resistant to the neurodegenerative loss associated with several diseases. In Huntington's disease up to 95 percent of neurons in an area called the caudate nucleus degenerate, but virtually no diaphorase neurons are lost. In vascular strokes and in some brain regions involved in Alzheimer's disease, diaphorase neurons are similarly resistant. Neurotoxic destruction of neurons in culture by NMDA, a model for stroke, can kill 90 percent of neurons, whereas diaphorase neurons are completely preserved.

Researchers have been mystified as to why diaphorase neurons survive these neurotoxic insults. Insights into this puzzle would have therapeutic implications for major neurological conditions, including stroke, Alzheimer's disease and Huntington's disease.

The newly discerned overlap between diaphorase neurons and cerebral neurons containing nitric oxide synthase was inspiring. Because the potential relation of nitric oxide to NADPH was important, we set to work immediately, joined by Hwang, Ted M. Dawson, a neurologist working in our laboratory, and Majid Fotuhi, a graduate student. Soon we were able to show that precisely the same neurons stain for nitric oxide synthase and diaphorase, both in the brain and in the peripheral tissues. We also explained why nitric oxide synthase activity accounts for diaphorase staining: the dye accepts electrons that normally are used to oxidize arginine to nitric oxide, creating a blue color.

Our finding, while exciting, seemed illogical. Clearly, something about nitric oxide synthesis makes neurons resist neurotoxic damage. Yet at the same time, nitric oxide was the result of glutamate activity, and glutamate is responsible for neurotoxicity. Knowing the molecular mechanism responsible for such resistance could resolve the paradox and lead to insights about why neurons lacking nitric oxide synthase die so readily during neurotoxicity.

A way out of this dilemma would be to propose that the neurons making nitric oxide release it and that the nitric oxide, because of its toxicity, kills adjacent neurons. We already knew that after stimulation by moderate levels of glutamate, nitric oxide provokes the formation of cyclic GMP in adjacent nerve cells. But perhaps in the presence of high levels of glutamate, nitric oxide–producing neurons behave like macrophages—that is, they release lethal amounts of nitric oxide. If this theory is correct, then one might expect inhibitors of nitric oxide synthase to prevent neurotoxicity.



NITRIC OXIDE SYNTHASE in this rat brain can be seen as the light-colored regions of the olfactory bulb, striatum (the region most affected in Huntington's disease), colliculi (where visual and auditory information is processed) and cerebellum.

To examine that question directly, Valina L. Dawson and Edythe D. London of the Addiction Research Center in Baltimore, together with Dawson and us, examined the neurotoxicity of NMDA in cultures of cerebral cortical neurons. In this model, developed by Dennis W. Choi, then at Stanford, NMDA is added to cultures made from brain cells of fetal rats. One day after being exposed to NMDA for only five minutes, up to 90 percent of the neurons are dead. This model reflects the neurotoxicity that occurs in vascular strokes.

In these cultures we observed that nitroarginine, a particularly potent and selective inhibitor of nitric oxide synthase, completely prevents the neurotoxicity elicited by NMDA. Removing arginine from the incubation mixture similarly protects the cells. Moreover, hemoglobin, which binds with and thereby inactivates nitric oxide, also inhibits the toxic effects.

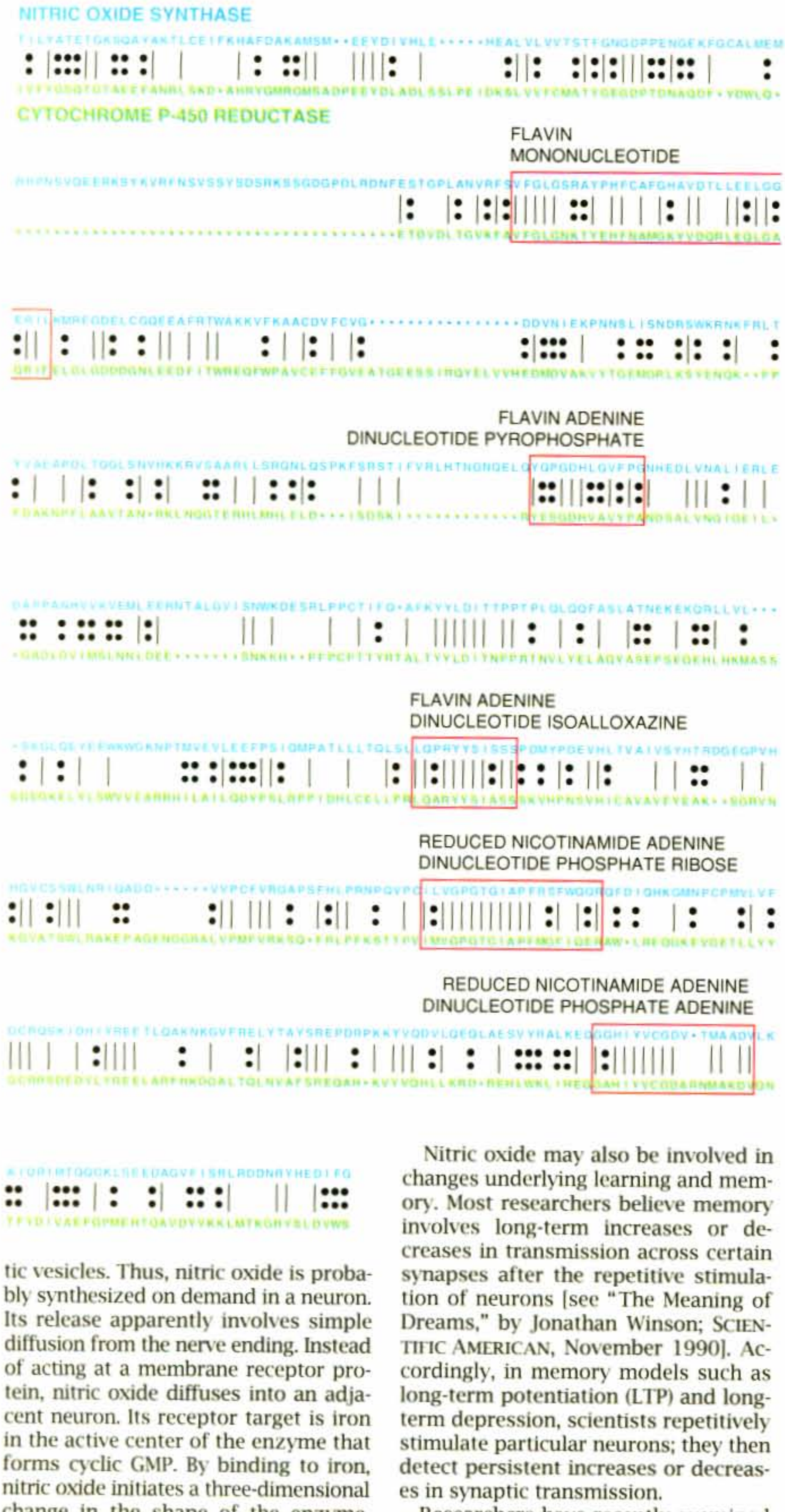
Thus, nitric oxide is clearly responsible for the neurotoxicity produced by glutamate acting at NMDA receptors in the cultures. Because NMDA antagonists can block the glutamate-induced damage associated with vascular strokes, nitric oxide may also modulate neuronal destruction caused by stroke.

Bernard Scatton and his colleagues at Synthelabo in Paris very recently injected small doses of nitroarginine into mice immediately after initiating a stroke. Nitroarginine reduced stroke damage by 73 percent; in a parallel experiment, however, a potent NMDA antagonist produced only 55 percent protection. These findings suggest that inhibitors of nitric oxide synthase may have therapeutic benefit in stroke and neurological damage associated with excess glutamate release.

The precise function of nitric oxide in neurons remains to be elucidated, and although we have made some progress, this mystery continues to unfold. Several lines of evidence indicate that nitric oxide acts very much like well-known neurotransmitters. In the intestines, for instance, the muscle relaxation involved in peristalsis is mediated by the myenteric plexus of nerves. But the neurotransmitter responsible for this relaxation had been elusive. Our demonstration that nitric oxide synthase is concentrated in these neurons suggested nitric oxide might be the missing messenger. Several laboratories have subsequently shown that the normal relaxation of muscle produced by electrically stimulating myenteric plexus neurons can be blocked by inhibitors of nitric oxide synthase. Moreover, direct addition of nitric oxide to the intestines mimics the effects of nerve stimulation.

If nitric oxide is a neurotransmitter, it is a most atypical one. Neurotransmitters are usually stable chemicals stored in synaptic vesicles in nerve terminals. They are released by a process in which the synaptic vesicle fuses with the neuronal membrane. On release, neurotransmitters interact with receptor proteins on the outside surface of the membranes of adjacent neurons.

In contrast, nitric oxide is not stored in vesicles. Together with Marcello Costa of Flinders University in Australia, we used electron microscopy to visualize nitric oxide synthase in myenteric plexus neurons. There the enzyme is located in the cytoplasm, not in synap-



AMINO ACID SEQUENCES deduced from segments of NOS and cytochrome P-450 reductase DNA suggest a common activity. Both have binding sites for cofactors (*red*), compounds assisting enzymes. Lines between amino acids indicate they are the same; two dots indicate they are very similar.

tic vesicles. Thus, nitric oxide is probably synthesized on demand in a neuron. Its release apparently involves simple diffusion from the nerve ending. Instead of acting at a membrane receptor protein, nitric oxide diffuses into an adjacent neuron. Its receptor target is iron in the active center of the enzyme that forms cyclic GMP. By binding to iron, nitric oxide initiates a three-dimensional change in the shape of the enzyme, which increases its activity and, consequently, the production of cyclic GMP. Because of this unique mode of action, nitric oxide represents a completely novel class of neuronal messenger.

Nitric oxide may also be involved in changes underlying learning and memory. Most researchers believe memory involves long-term increases or decreases in transmission across certain synapses after the repetitive stimulation of neurons [see "The Meaning of Dreams," by Jonathan Winson; SCIENTIFIC AMERICAN, November 1990]. Accordingly, in memory models such as long-term potentiation (LTP) and long-term depression, scientists repetitively stimulate particular neurons; they then detect persistent increases or decreases in synaptic transmission.

Researchers have recently examined the role of nitric oxide synthase in these long-term processes. Eric R. Kandel and his associates at Columbia University, Daniel Madison and his colleagues at Stanford and Georg Böhme and co-workers at Rhône-Poulenc Rorer in France independently studied the effects of nitric oxide synthase inhibitors. They determined the effects of the inhibitors on LTP in the hippocampus, a brain region known to be involved in memory. The researchers all found that the inhibitors blocked LTP. In addition, Katsuei Shibuki and Daisuke Okada of the Laboratory for Neural Networks in Japan showed such inhibition in the cerebellum.

We wondered whether nitric oxide might be only the first in a family of novel neuronal messengers. Some insights into this possibility have come from our molecular cloning of nitric oxide synthase. We performed the cloning with Hwang, Charles E. Glatt and Charles Lowenstein in our laboratory and in collaboration with Randall R. Reed of the Hopkins's Howard Hughes Medical Institute.

Once the amino acid sequence was established, we compared it with all known proteins. We found similarities to only one other known mammalian enzyme: cytochrome P-450 reductase. This finding was puzzling because cytochrome P-450 reductase had never been associated with the brain or with a neurotransmitter function.

Instead this enzyme serves as an electron donor for the P-450 enzymes in the liver. The P-450 enzymes metabolize a wide variety of drugs and, in some organisms, are set in motion by such environmental contaminants as dioxins and certain aromatic hydrocarbons. The electron-donating functions of cytochrome P-450 reductase are mediated by three cofactors that transfer electrons. Consequently, the enzyme's amino acid sequence has recognition sites for each of these three cofactors: NADPH, flavin adenine dinucleotide and flavin mononucleotide.

It is striking that nitric oxide synthase possesses recognition sites for the same three cofactors. Furthermore, the sites are located in the same places as they are in cytochrome P-450 reductase. There is also considerable similarity in the overall amino acid sequences of the two enzymes.

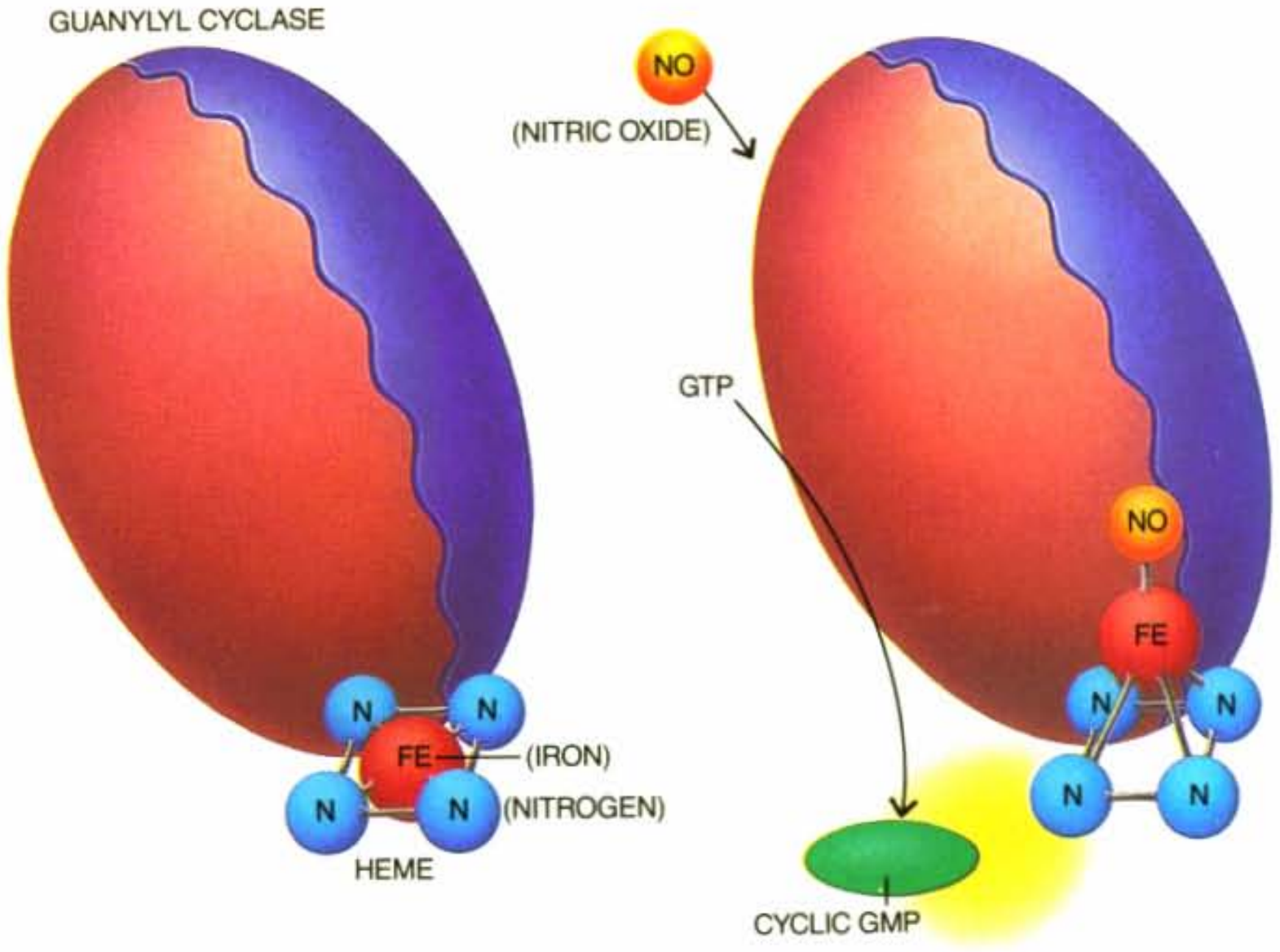
Nitric oxide synthase, however, has several other binding sites that are absent from cytochrome P-450 reductase.

It has a site for calmodulin and for phosphorylation. (Phosphorylation involves the addition of phosphate groups to proteins by a family of enzymes referred to as protein kinases.) The work of Paul Greengard of the Rockefeller University as well as many other researchers has established that protein phosphorylation relays information from second messengers, such as cyclic AMP, to various proteins inside cells. Phosphorylation is one of the major signaling mechanisms inside cells. We had noted earlier that nitric oxide synthase can be phosphorylated by a cyclic AMP–dependent protein kinase, a calcium-calmodulin–dependent protein kinase and an enzyme called protein kinase C. These kinases are important phosphorylating enzymes that regulate cellular responsiveness to hormones, neurotransmitters and growth factors.

Because nitric oxide synthase has so many sites for regulation, it is clearly influenced by more factors than are most other enzymes. This responsiveness makes sense when we consider the crucial role nitric oxide plays in providing subtle modulation for a number of processes. This function seems to contrast with the more mundane part performed by cytochrome P-450 reductase, despite the many similarities between their sequences.

What might these two enzymes have in common? Besides regulating drug-metabolizing enzymes, cytochrome P-450 reductase has one other function. It donates electrons to the enzyme heme oxygenase, which breaks down heme, the iron-containing oxygen-carrying constituent of red blood cells. The best-characterized form of heme oxygenase, called number one, is concentrated in the spleen, where red blood cells are normally degraded. But another, less understood form of the enzyme, number two, occurs at high levels in the brain. Because the presence of heme oxygenase number two overlaps so consistently with the presence of cytochrome P-450 reductase, it seems likely that donating electrons to number two is a major function of cytochrome P-450 reductase.

A close examination of this electron-donating process revealed what may well prove to be the second of the new class of neuronal messengers. During this process, heme oxygenase liberates carbon monoxide. Most of us know of carbon monoxide as a toxic gas from car exhaust. It binds to the hemoglobin and impairs its oxygen-transport activity. Carbon monoxide, however, is a normal body compound. By binding to the heme of guanylyl cyclase, the enzyme that produces the second messenger, cyclic GMP, it stimulates the formation of cyclic GMP, just as nitric oxide does.



NITRIC OXIDE RECEPTOR is iron in the enzyme guanylyl cyclase (*left*), which makes the second messenger, cyclic GMP. Nitric oxide binding (*right*) causes the iron-containing heme group to undergo a three-dimensional change that increases the production of cyclic GMP from GTP, a chemical with diverse functions.

Ajay Verma, a doctoral student in our laboratory, suggested that carbon monoxide was an excellent candidate to serve as a neuronal messenger. Together with David Hirsch, an undergraduate student, he rapidly amassed evidence favoring this notion. Verma and Hirsch showed that messenger RNA for heme oxygenase number two is situated in populations of neurons that closely resemble the distribution of both cytochrome P-450 reductase and guanylyl cyclase.

These findings are consistent with the possibility that carbon monoxide is the main regulator of cyclic GMP in the brain. When Verma and Hirsch treated cultures of brain neurons with an inhibitor of heme oxygenase, levels of cyclic GMP plummeted, but inhibitors of nitric oxide synthase had no effect. Thus, apparently carbon monoxide normally maintains levels of cyclic GMP in a number of brain regions. The similar structure of nitric oxide synthase and cytochrome P-450 reductase would seem to reflect their parallel roles in the synthesis of the two sister messengers: carbon monoxide and nitric oxide. Although the two compounds are found in different regions of the brain and appear to be involved in different pathways, their modes of action are very similar.

Whether or not carbon monoxide is another member of a new group of neurotransmitters, nitric oxide clearly is an extraordinary, novel and important messenger molecule. Small, short-lived and having strange ways, it appears to rival the major neurotransmitters in significance.

FURTHER READING

THE OBLIGATORY ROLE OF ENDOTHELIAL CELLS IN THE RELAXATION OF ARTERIAL SMOOTH MUSCLE BY ACETYLCHOLINE. Robert F. Furchgott and John V. Zawadzki in *Nature*, Vol. 288, No. 5789, pages 373–376; November 27, 1980.

NITRIC OXIDE FROM L-ARGININE: A BIOREGULATORY SYSTEM. Edited by S. Moncada and E. S. Higgs. Excerpta Medica, 1990.

CLONED AND EXPRESSED NITRIC OXIDE SYNTHASE STRUCTURALLY RESEMBLES CYTOCHROME P-450 REDUCTASE. David S. Bredt, Paul M. Hwang, Charles E. Glatt, Charles Lowenstein, Randall R. Reed and Solomon H. Snyder in *Nature*, Vol. 351, No. 6329, pages 714–718; June 27, 1991.

NITRIC OXIDE: PHYSIOLOGY, PATHOPHYSIOLOGY AND PHARMACOLOGY. S. Moncada, R.M.J. Palmer and E. A. Higgs in *Pharmacological Reviews*, Vol. 43, No. 2, pages 109–142; June 1991.

NITRIC OXIDE: A NOVEL NEURONAL MESSENGER. D. S. Bredt and S. H. Snyder in *Neuron*, Vol. 8, No. 1–20, pages 8–11; January 1992.