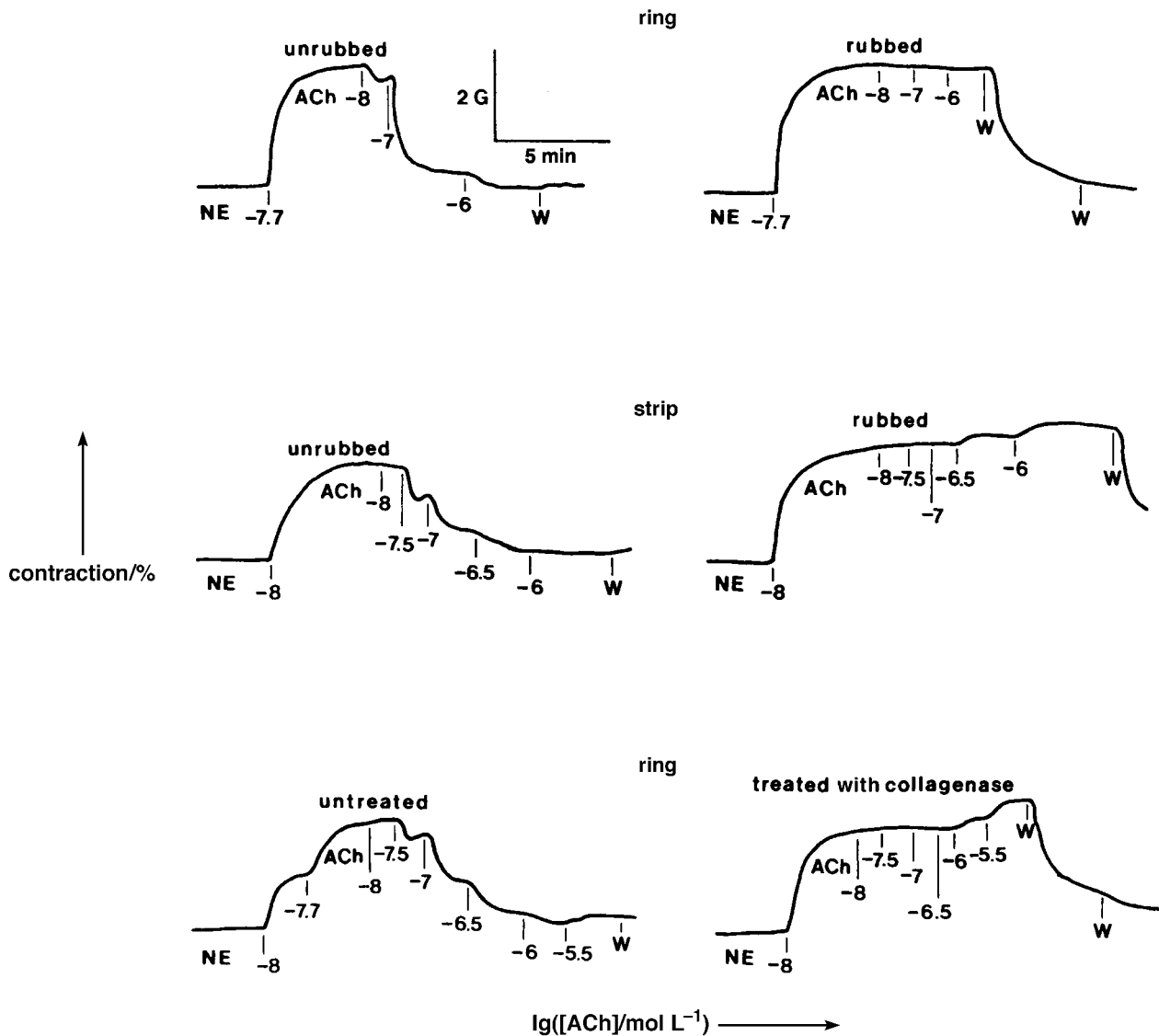


The obligatory role of endothelial cells in the relaxation of arterial smooth muscle is clearly shown with organ-chamber experiments. Two preparations, a transverse ring and a helical strip from the same rabbit thoracic aorta, were incubated in an organ bath and contracted with noradrenaline (NE).



Addition of the vasodilator acetylcholine (ACh) results in relaxation of the smooth muscle cells (left-hand side of the picture). If the endothelial cells are removed mechanically (by rubbing) or enzymatically (by exposure to collagenase), the relaxing response is eliminated (right-hand side of the picture). After washout (W) of NE and ACh, the muscle relaxes again.

Endothelium-Derived Relaxing Factor: Discovery, Early Studies, and Identification as Nitric Oxide (Nobel Lecture)**

Robert F. Furchgott

An Accidental Finding

In a paper published 45 years ago, in which I introduced the helical strip of the rabbit aorta as a useful preparation for quantitative pharmacological studies on vasoactive drugs and drug–receptor interactions, I reported on the responses of this preparation to a number of catecholamines, sodium nitrite, histamine, and acetylcholine (ACh). One somewhat surprising finding^[1] was that ACh, well recognized as a very potent vasodilator in whole animal and perfused organ studies, elicited no relaxation but only contraction of the aortic strip, whether or not the strip was tested at rest or precontracted with a vasoconstrictor like norepinephrine. Somewhat later, we used ACh as the contracting agent when determining the relative potencies of norepinephrine, epinephrine, and isoproterenol as relaxing agents acting through β -adrenoceptors on helical strips in which α -adrenoceptors had been irreversibly blocked by pretreatment with dibenamine.^[2] The relative potencies of these catecholamines as relaxants (approximately 1:50:200) indicated a β_2 -type adrenoceptor.

In the mid 1970s, in pharmacological studies on isolated strips of guinea-pig tracheal smooth muscle, we obtained evidence that the β -adrenoceptors of that muscle, which according to dogma were pure β_2 -type, frequently had a fairly high admixture of the β_1 -type with the β_2 -type. In view of this unexpected finding, I decided that it would be well to reexamine the type of β -receptor in the smooth muscle of rabbit thoracic aorta. The plan was first to determine anew in a series of experiments the relative potencies of norepinephrine, epinephrine, and isoproterenol in producing relaxation of preparations of rabbit aorta after dibenamine pretreatment. To produce contraction after the pretreatment, we planned to use a muscarinic agonist like carbachol or ACh, as in our earlier studies. However, in the very first experiment of this planned series in May 1978, my technician did not follow

my directions correctly: early in the experiment before, blocking the α -adrenergic receptors with dibenamine, he tested carbachol for its contracting activity before rather than after (as prescribed in the directions) washout of a previous test dose of norepinephrine. The response to carbachol was not a contraction, but was a partial relaxation of the norepinephrine-induced contraction. ACh was then tested. It too produced relaxation of the aortic preparation precontracted with norepinephrine (Figure 1). This was the first time

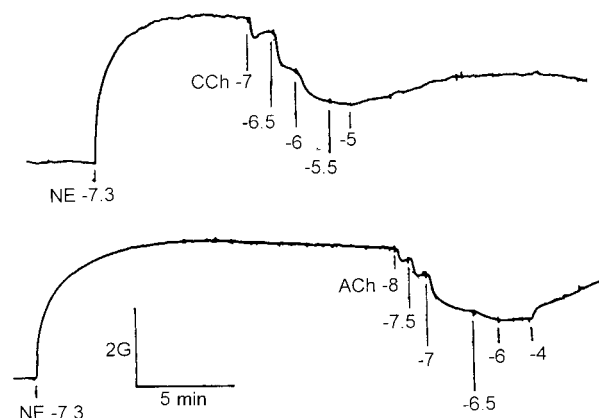


Figure 1. Polygraph tracings from the first experiment in my laboratory in which the muscarinic agonists, carbachol (CCh) and acetylcholine (ACh), unexpectedly produced relaxation of transverse rings of rabbit thoracic aorta. In this and all other experiments, unless otherwise noted, aortic preparations (rings or strips) were mounted in 20-mL all-glass organ chambers containing Krebs bicarbonate solutions aerated with O_2/CO_2 (95/5) and kept at 37°. Tracings show changes in tension under isometric conditions. (For details of experimental procedures, see reference [4].) In this and all other figures unless otherwise noted, cumulative concentrations of drugs in organ chambers are expressed as the log of molar concentrations. 2G = tension; NE = norepinephrine.

that I had ever observed relaxation of rabbit aorta in response to muscarinic agonists over the many years that I had been using this blood vessel for in vitro studies. The unexpected relaxation of rabbit aorta in vitro by muscarinic agonists was very exciting, for it was an effect that was in accord with the potent vasodilating action of these agonists in vivo.

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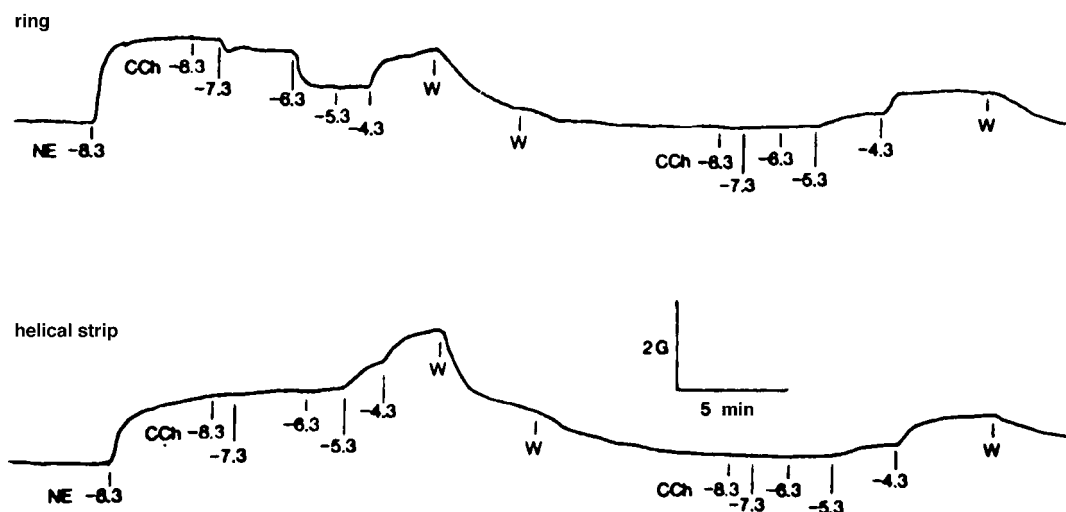


Figure 2. Comparison of responses to carbachol (CCh) of a transverse ring and a helical strip prepared from the same rabbit thoracic aorta. The ring (2.5 mm wide) was mounted on a pair of L-shaped hooks for attachment to the base of the organ chamber and to the force transducer. The strip, prepared by our standard method, had thread ties at each end for attachment. Only the ring, when precontracted with norepinephrine (NE), relaxed in response to low concentrations of CCh. It was subsequently recognized that in our standard method of preparing strips, the endothelial cells were unintentionally rubbed off the intimal surface. (There is no relaxation of the ring in response to CCh added after washout (W) of NE because such preparations exhibit practically no active contractile tension in the absence of vasoconstrictor agents.) Concentrations of drugs are expressed as the log of concentrations in g mL^{-1} . Reproduced with permission from reference [3].

Discovery and Early Studies on EDRF

Why had we failed to observe ACh-induced relaxation or aortic preparations in the past, and why did it now become manifest? A detailed account of our investigation of this matter has been published,^[3,62] so it will suffice here to outline briefly the sequence of findings. First, we were aware that the kind of preparation of rabbit thoracic aorta that we were now using was the transverse ring, rather than the helical strip which we had used in all of the earlier experiments with muscarinic agonists.

Several experiments were therefore carried out in which we compared the responses to cumulative concentrations of carbachol or ACh on helical strips and transverse rings prepared from the same aorta. Only the rings responded with

relaxation to these muscarinic agonists, while the strips responded with contraction (Figure 2).

After several weeks of work, we recognized that in any kind of preparation (ring or strip), gentle rubbing of the intimal surface, whether intentional or unintentional (as had occurred, unbeknown to us, in our standard method of preparing helical strips), eliminated the relaxing response to ACh and other muscarinic agonists. Strips as well as rings gave excellent relaxation when care was taken not to rub the intimal surface. That the loss of the relaxation response to ACh after intimal rubbing was the result of mechanical removal of the endothelial cells was clearly demonstrated with the use of a silver staining procedure to allow en face microscopy of the intimal surfaces. It was also demonstrated that complete removal of endothelial cells of the aorta by preincubation of

Robert F. Furchgott was born in Charleston, South Carolina. He received a BS degree in chemistry from the University of North Carolina in 1937 and a PhD degree in biochemistry from Northwestern University in 1940. He was at Cornell University College of Medicine from 1940–1949 and at Washington University from 1949–1956. He served as Professor



and Chairman of the Department of Pharmacology at the State University of New York Downstate Medical Center (now SUNY Health Service Center at Brooklyn) from 1956–1982, and is presently Distinguished Professor Emeritus at that institution. Since 1989 he has also been Adjunct Professor of Pharmacology at the University of Miami School of Medicine. His research interests have focussed on cardiac pharmacology, adrenergic peripheral mechanisms, theory of drug–receptor mechanisms, and vascular pharmacology and physiology. Much of his research has been carried out on isolated, living preparations of heart and blood vessels. The obligatory role of endothelial cells in the relaxation of arteries by acetylcholine and related muscarinic agonists and the demonstration that the relaxation resulted from release of a labile factor (EDRF) from the stimulated endothelial cells are among the important discoveries he has made. His outstanding contributions have been recognized by numerous prestigious awards and honors, culminating in 1998 with the Nobel Prize in Physiology or Medicine.

the intimal surface with collagenase also resulted in a complete loss of the relaxation response to ACh.

Figure 3 is a slightly modified figure from the first full paper on the obligatory role of endothelial cells in the relaxation of arterial smooth muscle by ACh.^[4]

In that paper, we also reported that in addition to the thoracic aorta, a number of other arteries from the rabbit and various arteries from other species of laboratory animals also exhibited relaxation in response to ACh only if endothelial cells were present. Cyclooxygenase inhibitors did not interfere with the endothelium-dependent relaxation. One hypothesis to explain the obligatory role of endothelial cells was that ACh acting on a muscarinic receptor of these cells stimulates them to release a non-prostanoid substance that diffuses to and activates relaxation of the subjacent smooth muscle cells. Direct evidence for this hypothesis was obtained with the so-called "sandwich" procedure in which it was shown that a transverse strip of aorta freed of endothelial cells, and therefore unable to relax on exposure to ACh, could be made to relax in response to ACh if it were remounted for recording with its endothelium-free intimal surface placed against the endothelium-containing intimal surface of a longitudinal strip of aorta (Figure 4). The ACh-induced relaxation of the transverse strip in the "sandwich" demonstrated that ACh stimulated the release of a diffusible relaxing substance (or substances) from the endothelial cells of the longitudinal strip.^[4] The relaxing substance was later referred to as EDRF (for endothelium-derived relaxing factor).^[5]

It should be noted here that although we had never observed relaxation of rabbit aortic preparations by ACh in my laboratory prior to our accidental finding in 1978, there had been occasional reports from other investigators of relaxation of isolated preparations of arteries in response to that agent. For example, Jelliffe^[6] had reported that a chain of thread-tied rings of rabbit aorta precontracted with serotonin relaxed in response to low concentrations of ACh; and Vanhoutte^[7] and Toda^[8] had found that on a number of canine arteries prepared as strips, ACh elicited significant relaxation of spontaneous or drug-induced contractions. After our findings on the obligatory role of endothelial cells in ACh-induced relaxation of arteries, it became apparent that these earlier examples of ACh-induced relaxation were dependent on the presence of endothelial cells in the preparations of the arteries being studied.

It is of some interest that an unintentional removal of endothelial cells in another vascular preparation used in my laboratory may have prevented the discovery of endothelium-dependent vasodilation at an earlier date. That preparation was the perfused rabbit central ear artery which Odd Stein-land, while a graduate student in my laboratory in the early 1970s, used for investigating the inhibition by ACh and other muscarinic agonists of adrenergic neurotransmission by their action on prejunctional muscarinic inhibitory receptors. He readily demonstrated that ACh was a potent inhibitor of neurotransmission, as evidenced by its inhibition of both the release of norepinephrine and the vasoconstriction in re-

sponse to nerve stimulation.^[9] To make sure that the inhibition of the vasoconstriction was only due to inhibition of transmitter release, he tested whether infused ACh could inhibit vasoconstriction produced by infused norepinephrine, and found that it did not.^[9] About ten years later, after endothelium-dependent relaxation by ACh had been found in a large variety of arterial preparations, we were puzzled by the earlier finding that the perfused ear artery constricted with infused norepinephrine had failed to show any vasodilation in response to ACh. Dr. Stein-land provided us with the answer. In his earlier work, he had not taken precautions to prevent air bubbles from entering the perfusion line and passing through the ear artery. When care was now taken to avoid the occurrence of bubbles in the perfusion stream through the artery, ACh elicited good vasodilation (relaxation) of norepinephrine-induced vasoconstriction.^[10] Apparently, in the earlier work, the bubbles had mechanically removed the endothelial cells, and we had been unaware of this action.

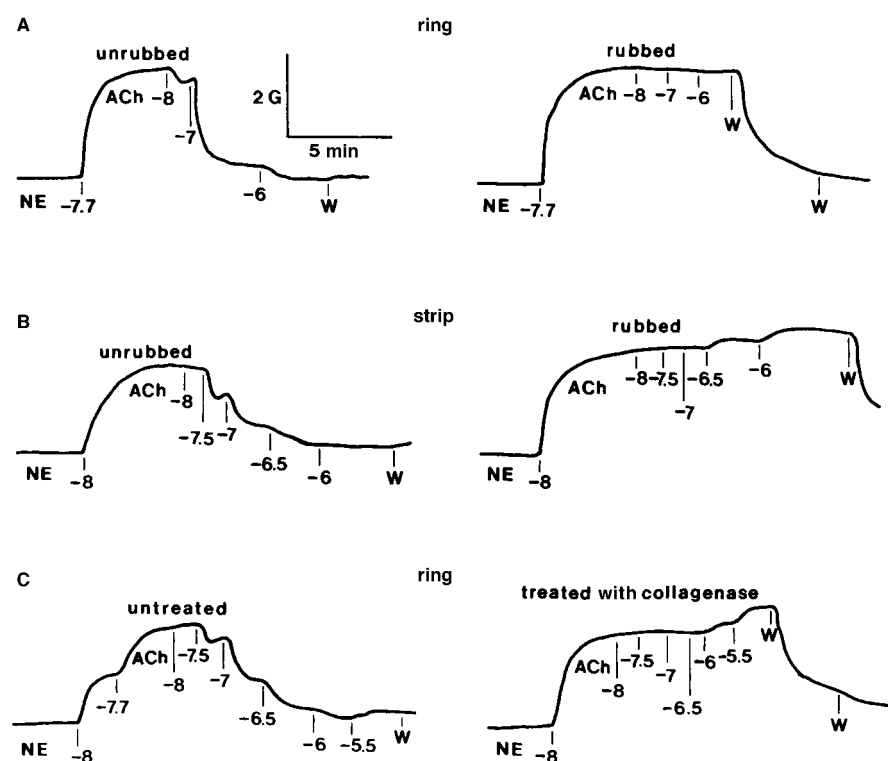


Figure 3. Recordings showing loss of relaxing response of rabbit aorta to ACh after complete removal of endothelial cells. In each case, the recording on the left was made before removal and that on the right after removal of endothelial cells. The cells were removed from the ring in A by rubbing its intimal surface with a small wooden stick, from the transverse strip in B by rubbing its intimal surface on moistened filter paper, and from the ring in C by exposing its intimal surface to collagenase. After the procedure to remove endothelial cells, each preparation was allowed to reequilibrate in its organ chamber for about 1 h before being retested. Reproduced with permission from reference [18].

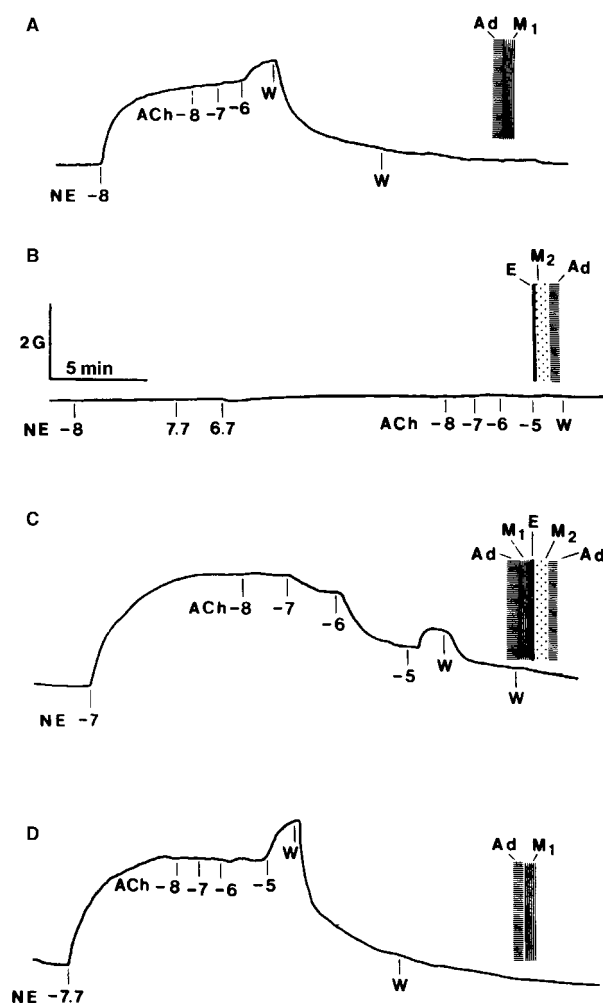


Figure 4. "Sandwich" experiment demonstrating release of a relaxing substance from endothelial cells in response to ACh. A: On a transverse strip of rabbit aorta with endothelial cells removed by rubbing (first test); ACh failed to relax the NE-induced tone. B: On a longitudinal strip from the same aorta with endothelium present (first test); NE and ACh produced only trivial changes in tension because of the orientation of the smooth muscle cells. C: On a "sandwich" made from the transverse strip without endothelium and the longitudinal strip with endothelium mounted together in clips with their entire intimal surfaces apposed, and allowed to reequilibrate (second test); ACh now produced relaxation of the NE-induced tone. D: On the transverse strip after removing the longitudinal strip with endothelium (third test); after reequilibration there was again no relaxation in response to ACh. Insets: diagrams of the vertical cross-sections of the preparations; Ad = adventitia, E = endothelial cell layer, M₁ and M₂ = medial layers of smooth muscle cells oriented vertically and horizontally, respectively. Reproduced with permission from reference [18].

Early Studies on Agents Inducing and Inhibiting Endothelium-Dependent Relaxation

Within a few years after the discovery that ACh-induced relaxation of isolated arteries was endothelium-dependent, a number of other endothelium-dependent relaxing agents were identified in organ-chamber experiments using rings or strips of blood vessels from a variety of species, including man. Among these were the calcium ionophore A23187, ATP and ADP, substance P, bradykinin, histamine, thrombin, serotonin,

and vasopressin. (For references to first reports on endothelium-dependent relaxation by these and other agents, see references [11, 12].) The early finding that the calcium ionophore A23187 was a very potent endothelium-dependent relaxing agent on all vessels tested suggested a central role for calcium in the release, or synthesis and release, of EDRF by endothelium-dependent relaxing agents.^[13]

It was soon recognized in this early work that, depending on the agent, endothelium-dependent relaxation might be limited to certain species, or even to specific arteries from a given species. For example, whereas ACh relaxed by this mechanism practically all systemic arteries from all mammalian species tested, histamine, another potent vasodilator, produced endothelium-dependent relaxation of rat^[14] but not of rabbit arteries.^[15] Another example was bradykinin, which gave endothelium-dependent relaxation of isolated canine and human arteries but not of rabbit or cat arteries.^[5] As examples of vessel specificity, it was found that noradrenaline activated endothelium-dependent relaxation in isolated canine coronary arteries by acting on α_2 -adrenoceptors of the endothelial cells, but failed to activate this kind of relaxation in canine systemic arteries,^[16] and that serotonin activated endothelium-dependent relaxation in canine coronary arteries but not in canine systemic arteries.^[16, 17]

Among the conditions and agents which were found to inhibit endothelium-dependent relaxation of arteries in the early organ-chamber experiments were anoxia and some agents that were recognized as inhibitors in the metabolic pathway of arachidonic acid (AA) from phospholipids through lipoxygenase.^[4, 18] Because it had already been reported that in certain smooth muscles there was a positive relationship between an increase in cyclic guanosine monophosphate (cGMP) and relaxation^[19–22] and that guanylate cyclase (G-cyclase) was markedly stimulated by hydroperoxides of AA,^[23, 24] I speculated 1) that EDRF is either a short-lived hydroperoxide or free radical arising as an intermediate product in the oxidation of liberated AA by the lipoxygenase pathway, and 2) that EDRF stimulates the G-cyclase of the vascular smooth muscle, causing an increase in cGMP which then somehow activates relaxation.^[18] As it turned out, this early speculation about the nature of EDRF was wrong, but that about the role of cGMP proved to be right.

Cyclic GMP as a Mediator for EDRF and Nitrovasodilators

The speculation that EDRF released by ACh would stimulate an increase in cGMP was soon proven correct in a number of independent studies by, for example, Rapoport and Murad^[25] using rat aorta, Diamond and Chu^[26] and Furchgott et al.^[27] using rabbit aorta, Holzmann^[28] using bovine coronary artery, and Ignarro et al. using bovine pulmonary artery.^[29] All of these investigators showed that a rise in cGMP accompanied the ACh-induced relaxation of the blood vessel, consistent with the proposal that the increase in cGMP in the muscle has a causal role in the relaxation (Figure 5). Endothelium-dependent relaxation of rat aorta by A23187

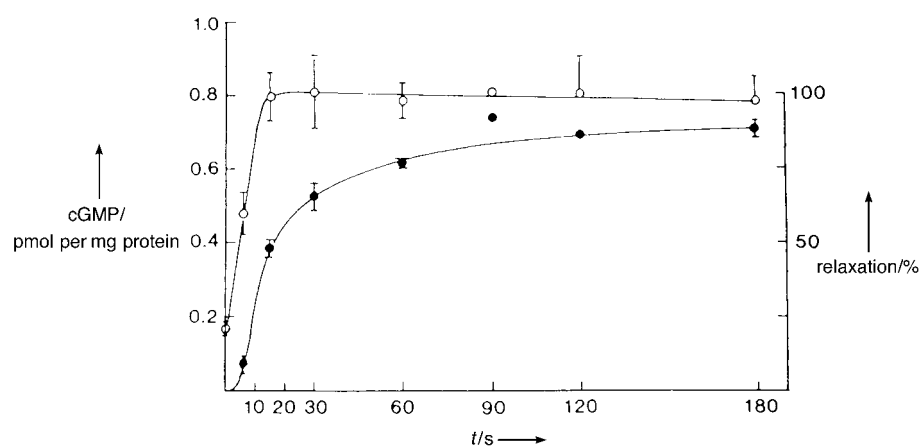


Figure 5. Time course of changes of cyclic GMP (cGMP, \circ) and of relaxation (\bullet) of endothelium-containing rings of rabbit thoracic aorta after addition of ACh. Norepinephrine ($0.1 \mu\text{M}$) was first added to produce a steady contraction. ACh ($1 \mu\text{M}$) was then added, and the tissue was instantly frozen in liquid nitrogen after the desired period of exposure to ACh. The level of cyclic AMP (not shown) did not change significantly during the endothelium-dependent relaxation. Reproduced with permission from reference [27].

and by histamine^[25] and of rabbit aorta by A23187^[27] was also accompanied by a rise in cGMP.

It is well to stress at this point that Murad and co-workers^[21, 22] had previously found that nitric oxide (NO) is a very potent stimulus of G-cyclase, and had proposed that a number of agents (e.g. glyceryl trinitrate, nitroprusside, azide), which they and others had found to increase cGMP and produce relaxation of certain non-vascular smooth muscle preparations, do so by generating NO as the proximal activator of G-cyclase. Also, Ignarro and his co-workers^[30] had found that the relaxation of a vascular preparation (bovine coronary artery) by NO as well as by nitroprusside was associated with an increase in cGMP. However, despite these early findings with NO, none of the investigators who several years later showed that endothelium-dependent relaxation of arteries in organ-chamber experiments was also correlated with an increase in cGMP^[30] hypothesized that EDRF might be the free radical NO. Certain key findings had yet to be made to foster this novel proposal.

Further Studies on EDRF, Including Those with Perfusion – Bioassay Procedures

Hemoglobin and Methylene Blue as Inhibitors

Two agents that became widely used as inhibitors of endothelium-dependent relaxation were hemoglobin (Hb) and methylene blue (MB). Hb had been found by Murad et al.^[21] to inhibit guanylate cyclase in cellular extracts, and by workers at Glasgow University^[31] to inhibit the relaxation of strips of bovine retractor penis (BRP) muscle in response either to nonadrenergic, noncholinergic (NANC) nerve stimulation or to an acid-activated extract of that tissue. Gruetter et al.^[32] had reported that methemoglobin (metHb) interfered with NO-induced increases in vascular cGMP. In our laboratory, work with Hb began in 1983 when William

Martin, who had studied the properties of extracts of the BRP muscle while a graduate student at Glasgow University,^[33, 34] came to my laboratory as a postdoctoral fellow (see below). It was soon found that Hb was an extremely potent and fast-acting inhibitor of endothelium-dependent relaxation and of the associated rise in cGMP induced by ACh, A23187, and other agents on rabbit and rat aorta.^[27, 35, 36] Myoglobin was as effective as Hb, but metHb only inhibited very weakly^[37] (Figure 6).

The degree of potentiation by Hb of contractions of arterial rings by vasoconstrictors such as phenylephrine also provided a measure of the relaxing activity of basally released EDRF.^[36] Using this measure,

the relaxing activity of basally released EDRF in the case of rings of rat aorta appeared to be considerably greater than in the case of rings of rabbit aorta. With rings from both species, addition of phosphodiesterase inhibitors (such as zaprinast and IBMX) produced relaxation associated with increases in cGMP.^[38] The responses to these inhibitors of the enzymes which hydrolyze cGMP to GMP were more pronounced in the case of rat aorta than in rabbit aorta, consistent with a higher basal release of EDRF and higher basal stimulation of G-cyclase in the former. The relaxation of endothelium-containing rings of both species produced by phosphodiesterase inhibitors could be completely blocked by Hb.

It was originally proposed that Hb inhibits relaxation and the rise in cGMP by binding EDRF as it diffuses from the endothelial cells, thus preventing it from reaching and activating the G-cyclase in the muscle cells.^[35] However, after the identification of EDRF as NO, it became apparent that the scavenging of EDRF by Hb under the usual aerobic conditions was the result of the very rapid reaction of HbO₂ with NO to form NO₃⁻ and metHb, as first reported by Doyle and Hoekstra.^[39]

Methylene blue had first been shown by Gruetter et al.^[32] to inhibit stimulation of G-cyclase by nitrovasodilators in cell-free systems and to inhibit both the relaxation and increase in cGMP elicited by these agents in vascular smooth muscle. Subsequently, MB was found to inhibit both the endothelium-dependent relaxation and accompanying increase in cGMP produced by ACh in bovine arteries^[28, 29] and by ACh and A23187 in rabbit aorta.^[27, 35] Holzman^[28] and Ignarro et al.^[29] concluded that the inhibition of relaxation was the result of the inhibition of G-cyclase by the MB. On the other hand, I and my co-workers, although agreeing that such enzyme inhibition accounted for the sustained inhibition by MB developed during prolonged exposure of the rabbit aorta, proposed that the extremely fast inhibition of endothelium-dependent relaxation immediately after addition of MB to an organ chamber (as in Figure 6) resulted from some more

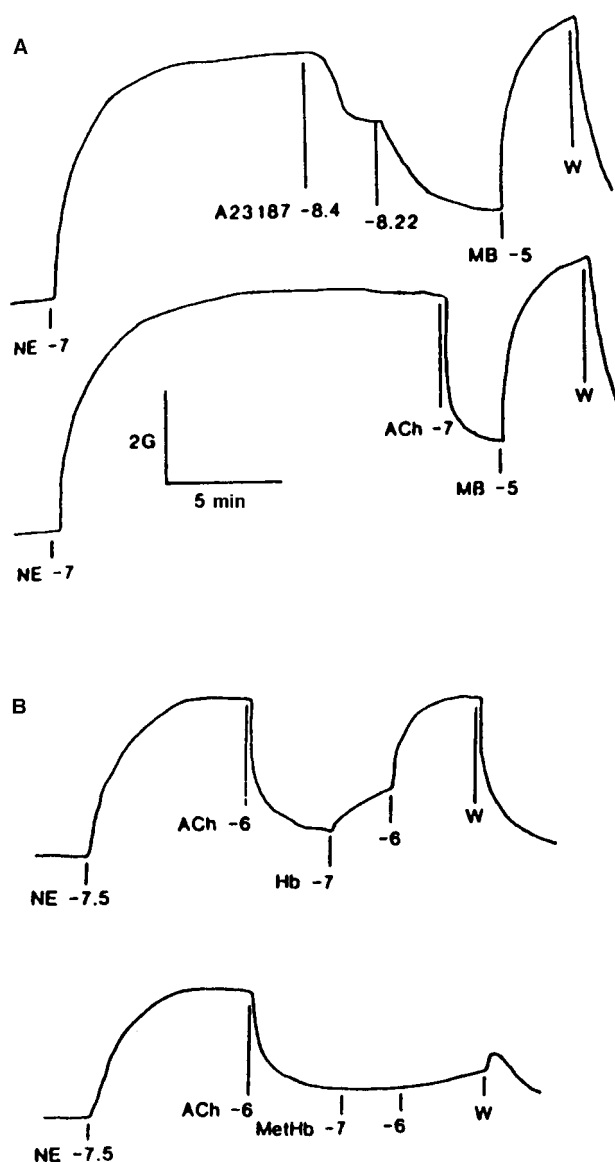


Figure 6. Examples of inhibition by methylene blue (MB) and by hemoglobin (Hb) of endothelium-dependent relaxation in rings of rabbit aorta. A: Addition of MB ($10\mu\text{M}$) rapidly and completely inhibited the ongoing relaxation produced by addition of ACh (top) and that produced by addition of the calcium ionophore A23187 (bottom). B: Addition of Hb ($1\mu\text{M}$) rapidly and almost completely inhibited the relaxation produced by addition of ACh (top); however, addition of methemoglobin (MetHb) at the same concentration inhibited ACh-induced relaxation only to a very small extent (bottom).

direct inactivation of EDRF by the MB.^[27, 35] It now appears that this more direct inactivation is due to superoxide anions, generated by reactions of the MB in the tissue.^[40]

Results with Perfusion–Bioassay Procedures

For more direct studies on the properties of EDRF than were possible in organ-chamber experiments, perfusion–bioassay procedures were developed. These procedures, although first developed in laboratories other than my own, should be considered here, because they produced results

which contributed much to the identification of EDRF. In such procedures, endothelial cells are upstream in the perfusion (or superfusion) cascade and an endothelium-denuded arterial preparation (usually a ring or one or more strips), whose contractile state is monitored, is downstream to bioassay EDRF in the perfusion fluid. In some procedures, the endothelial cells upstream were native cells on the luminal surface of a perfused artery;^[41–44] in other procedures, they were cultured cells (usually from bovine or porcine aortas) on the surface of microcarrier beads contained in a perfused column.^[45, 46]

Perfusion–bioassay procedures allowed one to determine clearly whether an inhibitor of endothelium-dependent relaxation interfered with the synthesis/release of EDRF or inactivated the EDRF after its release. In addition, by altering the transit time of the perfusate between the endothelial cells and the bioassay preparation, it was possible to study the rate of decay of EDRF. Surprisingly, estimated half-life values for EDRF ranged from as low as 4–6 s to as high as 50 s depending on the biological preparations and the experimental conditions. The likely explanation of these discrepant values came with the important finding that superoxide dismutase (SOD) markedly stabilizes EDRF released from either endothelial cells on arteries^[47] or cultured endothelial cells on microcarrier beads,^[46] clearly showing that the superoxide anion (O_2^-) very rapidly inactivates EDRF. Thus, the marked differences in half-life of EDRF reported from different laboratories could largely be attributed to differences in concentrations of O_2^- in the perfusion fluid leaving the endothelial cells under the different experimental conditions used. Subsequently, Moncada et al.^[48] were able to show with their perfusion–bioassay procedure that inhibition of endothelium-dependent relaxation by certain reducing agents (e.g. hydroquinone, phenidone, and pyrogallol) originally demonstrated in organ-chamber experiments was principally the result of inactivation of EDRF by O_2^- formed in a single-electron oxidation of the reducing agent by O_2 .

Development of perfusion–bioassay procedures also led to the important finding that increases in shear stress on endothelial cells of the perfused artery with increases in rate of flow of the perfusion fluid resulted in increases in the rate of release of EDRF.^[49, 50] Increase in flow through arteries both in situ^[49, 51, 52] and in vitro produced vasodilation that could not be blocked by inhibitors of cyclooxygenase, but was abolished by removal of the endothelium. These findings concerning increased EDRF release with increased shear stress were important for they provided evidence that EDRF has a continuous physiological role in the control of regional blood flow and regulation of blood pressure.

Findings That Led to the Identification of EDRF as NO

While Billy Martin was working in my laboratory (1983–1985), I became interested in the work that he and others had done in John Gillespie's laboratory in Glasgow in trying to identify the neurotransmitter released from NANC (non-adrenergic, non-cholinergic) nerves that elicit relaxation of

bovine retractor penis (BRP) muscle and certain other smooth muscle preparations. I learned that relaxation produced by NANC nerve stimulation and relaxation produced by a partly purified extract made by Martin from the BRP muscle (referred to as BRP inhibitory factor or BRPIF) were both inhibited by Hb^[31] and were both associated with increases in cGMP in the muscle.^[53] One important aspect of Martin's work with BRPIF extract was that it had to be acidified for a short period prior to testing in order for it to have a relaxing effect on smooth muscle preparations of retractor penis and arteries in organ baths.^[33] Once activated by acidification, the BRPIF extract gave good relaxations of the smooth muscle preparations, but the relaxations were transient, lasting no more than a few minutes.

As discussed in the paper that I presented at a symposium in Rochester, Minnesota in July 1986,^[54] I was struck by the similarities of the transient relaxations produced by the acid-activated BRPIF extract of Martin and the transient relaxations of rabbit aorta produced by acidified solutions of sodium nitrite (NaNO₂). That acidified, but not neutral, solutions of NaNO₂ produce strong transient relaxations of rabbit aorta had been found about 15 years earlier in my laboratory when a new postdoctoral fellow, assigned to obtain cumulative dose-response curves for NaNO₂, accidentally made his standard solutions of that salt with acidified diluting fluid (used in my laboratory for making stable standard solutions of catecholamines) rather than with neutral diluting fluid (Figure 7). At

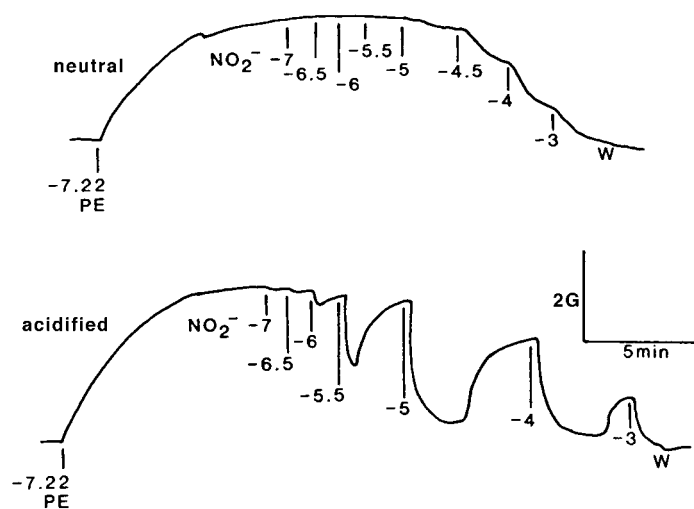


Figure 7. Comparison of the relaxing effects of cumulative additions of neutral and acidified solutions of sodium nitrite on rings of rabbit aorta. In this typical experiment, two rings from the same aorta after contraction with phenylephrine (PE) were exposed to additions of NaNO₂ diluted with neutral physiological saline and to additions of NaNO₂ diluted with the saline acidified with 11 mM HCl. The concentrations shown are the final nitrite concentrations (log of the molar concentration) in the organ chambers. Note the striking transient relaxations in the case of the acidified nitrite solutions. The rings in this experiment were denuded of endothelial cells, but results were essentially the same when endothelial cells were present. Reproduced with permission from reference [54].

that time, I became aware that the HNO₂ ($pK_a=3.2$) produced by acidification of NaNO₂ solutions immediately generates low concentrations of NO and NO₂ as a result of a reversible dismutation according to Equations (1) and (2).



However, when a solution of acidified nitrite is added to the oxygenated, well-buffered physiological salt solution used in our organ chambers, any NO present will be removed very rapidly—thus accounting for the transiency of the relaxation of the aortic rings receiving acidified nitrite.

In the spring of 1986, recalling the much earlier accidental finding in my laboratory with acidified sodium nitrite solutions, I began to consider the possibility that Martin's acid-activatable BRPIF was inorganic nitrite. An encouraging early finding was that the degree of transient relaxation as a function of pH of the added NaNO₂ solution in the case of rabbit aorta was similar to that found by Martin for his added extract in the case of the BRP—maximal transient relaxation at a pH of 2 or lower, and approximately half-maximal transient relaxation at a pH of 3.

By the time I had begun to use acidified nitrite solutions as a source of NO in experiments with rabbit aorta in 1986, I had also become aware of the recent findings by Gryglewski, Palmer and Moncada^[46] and by Rubanyi and Vanhoutte^[47] demonstrating that the superoxide anion (O₂⁻) rapidly inactivates EDRF and that superoxide dismutase (SOD) protects against this inactivation. Assuming that the free radical NO might rapidly react with the free radical O₂⁻, to form inactive NO₃⁻, I began to speculate on the possibility that EDRF is NO. (Later, it was pointed out by Beckman et al.^[55] that the immediate product of the reaction between NO and O₂⁻ is peroxynitrite (ONOO⁻), which is protonated to HONOO ($pK_a=6.8$), which decays within seconds at physiological pH.)

My speculation in the spring of 1986 that EDRF might be NO led to a large number of experiments on rabbit aorta, carried out with the assistance of D. Jothianandan and M. T. Khan, comparing the characteristics of relaxation by NO (present in acidified solutions of NaNO₂) with the characteristics of relaxation by EDRF (released by ACh). It was found that relaxation by NO, like that produced by EDRF, was strongly inhibited by Hb, strongly inhibited by MB, strongly inhibited by superoxide generators (e.g. hydroquinone, phenidone, ferrous sulfate), and potentiated by SOD when superoxide was limiting the degree of relaxation (Figure 8). It was already known that relaxation of vascular smooth muscle by NO, like that by EDRF, is accompanied by an increase in cGMNP. On finding the characteristics of NO and EDRF so similar, I had no hesitancy in proposing in the paper which I delivered at the July 1986 symposium that EDRF is NO.^[54] (Shortly after the symposium, we began using solutions of NO gas rather than acidified nitrite as a source of NO in our experiments and obtained identical results (Figure 9).) I also proposed that the acid-activatable inhibitory factor in the BRP extract of Martin and Gillespie is inorganic nitrite, and that the possibility had to be considered that the neurotransmitter of NANC nerves might also be NO.^[54]

At the same symposium at which I proposed that EDRF is NO, Ignarro, on the basis of studies in his laboratory on isolated bovine pulmonary arteries, independently made the

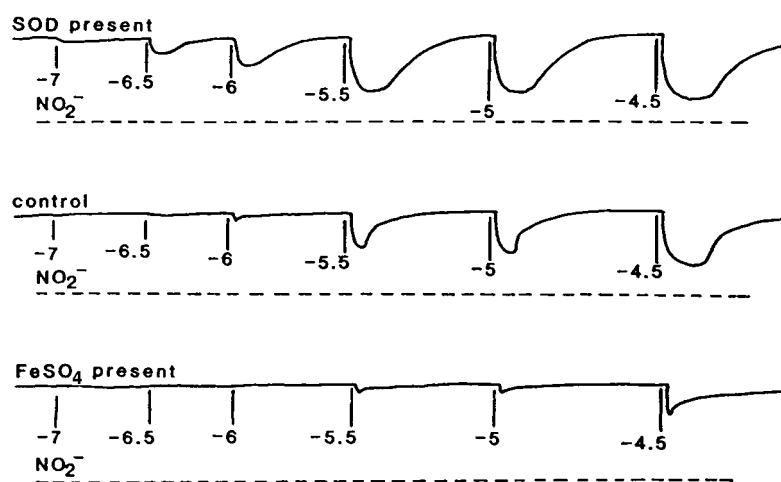


Figure 8. Transient relaxations of endothelium-free rings of rabbit aorta by nitric oxide present in acidified solutions (pH 2) of NaNO_2 , potentiation of the relaxation by SOD (25 units per ml), and inhibition by the superoxide generator FeSO_4 ($3 \times 10^{-4} \text{ M}$). Three matched rings from one aorta were contracted above basal tone (dashed line) with phenylephrine. SOD and FeSO_4 were added about 5 min before first additions of the acidified nitrite. Reproduced with permission from reference [54].

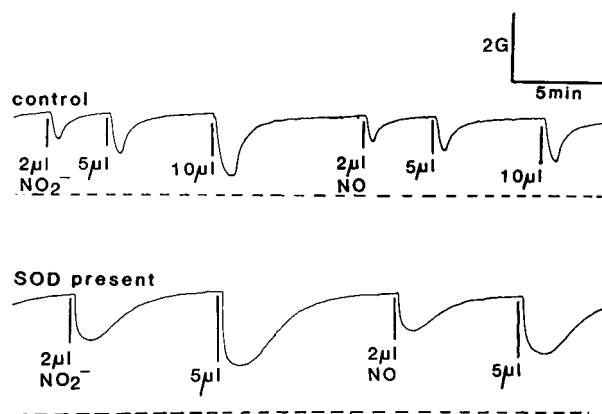


Figure 9. Similarity of the relaxing effects of a solution of acidified sodium nitrite and a solution of nitric oxide gas on rings of rabbit aorta in the absence and presence (30 units per ml) of SOD; 2.5 mM acidified nitrite (pH 2.0), $15 \mu\text{M}$ NO. (See reference [60] for a method for making solutions of NO.) Additions were to 20-mL aliquots of Krebs solution in each organ chamber, so that $2 \mu\text{L}$ of the NO solution gave an initial concentration of 1.5 nM . Both rings were precontracted with phenylephrine. The potentiation by SOD of the transient relaxations produced by the NO in the acidified nitrite and in the NO solution indicates that there was a considerable endogenous production of the superoxide anion O_2^- in the organ chambers.

same proposal. [56] (Unfortunately, the papers presented at that symposium were delayed in publication until 1988.) Soon after the 1986 symposium, three laboratories utilized perfusion–bioassay procedures to compare more accurately the biological and chemical characteristics of EDRF and NO. Ignarro and co-workers using bovine pulmonary vessels, [57] Moncada and co-workers using cultured porcine aortic endothelial cells, [58] and Khan and myself using rabbit aorta as a source of EDRF [44] all found EDRF released upstream and NO infused upstream to have similar rates of decay, similar susceptibility to inhibitors like Hb and superoxide generators, similar stabilization by SOD, etc. In addition,

Ignarro's laboratory presented spectroscopic evidence that the product of the reaction of released EDRF and Hb was the same as that of NO and Hb, and Moncada's laboratory showed that the amount of NO released by bradykinin (as determined by a chemiluminescence assay) could fully account for the relaxation of the bioassay strip by the EDRF released by the peptide. A year later, Moncada and co-workers made the major finding that the source of endothelial NO was a guanidinium nitrogen atom of L-arginine and that the enzyme responsible for its formation was an oxygenase (now called endothelial nitric oxide synthase or eNOS). [59] These findings marked the beginning of a major worldwide expansion of research on the role of NO in vascular physiology and pathophysiology.

Despite the evidence for the identity of EDRF and NO, some results obtained in various laboratories in the late 1980s did not appear to be completely consistent with that conclusion. [60]

Nevertheless, as of now, it has become generally accepted that EDRF is either NO, or some adduct that readily releases NO, or perhaps a mixture of NO and some adduct of NO. This does not preclude a contribution to endothelium-dependent relaxation in some blood vessels from the release of a non-nitric oxide endothelium-derived hyperpolarizing factor (EDHF) along with EDRF. [61]

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