Myocardial protection by nitrite

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Nitrite has long been considered to be an inert oxidative metabolite of nitric oxide (NO). Recent work, however, has demonstrated that nitrite represents an important tissue storage form of NO that can be reduced to NO during ischaemic or hypoxic events. This exciting series of discoveries has created an entirely new field of research that involves the investigation of the molecular, biochemical, and physiological activities of nitrite under a variety of physiological and pathophysiological states. This has also led to a re-evaluation of the role that nitrite plays in health and disease. As a result there has been an interest in the use of nitrite as a therapeutic strategy for the treatment of acute myocardial infarction. Nitrite therapy has now been studied in several animal models and has proven to be an effective means to reduce myocardial ischaemia-reperfusion injury. This review article will provide a brief summary of the key findings that have led to the re-evaluation of nitrite and highlight the evidence supporting the cardioprotective actions of nitrite and also highlight the potential clinical application of nitrite therapy to cardiovasculardiseases.

1. Introduction

Nitrite is an oxidative breakdown product of nitric oxide (NO) that has traditionally been viewed as an acute marker of NO flux/formation in biological systems. In this regard, nitrite has long been considered to be an inert by-product of NO metabolism. More recently, nitrite has moved to the forefront of NO biology with the discovery that it represents an important storage form of NO in blood and tissues that can be readily reduced to NO under certain pathological conditions. This exciting series of discoveries has established a new field of research that entails the investigation of the molecular, biochemical, and physiological activities of nitrite under a variety of physiological and pathophysiological states. Among the most exciting developments in the field of nitrite physiology is the recent finding that nitrite is cytoprotective in a number of animal models of disease, including myocardial ischaemia-reperfusion (I/R) injury. In this review we will provide a brief summary of the key findings that have led to the re-evaluation of the biochemical and physiological role of nitrite, as well as, highlight the most recent evidence supporting the cardioprotective actions of nitrite. Finally, we will also evaluate the therapeutic potential of nitrite therapy for cardiovascular diseases such as acute myocardial infarction (MI), congestive heart failure, and peripheral artery disease.

2. Nitric oxide and cardioprotection

Although the mechanisms of nitrite-mediated cardioprotection have not been fully elucidated, there is a consensus that NO plays a prominent role. Therefore, we will begin this article with a brief historical account of NO and cardioprotection. NO is a member of a family of labile biological mediators termed gasotransmitters. It was first discovered in the late-1700s and, for the better part of 200 years, was widely considered to be a toxic gas and environmental hazard. The importance of NO in the field of biology and medicine was not fully appreciated until the 1980s when it was discovered that NO is generated in mammals, including humans, by nitric oxide synthases (NOSs) and plays a prominent role in controlling blood pressure via the regulation of vascular tone. There are three isoforms of NOS that have been characterized, purified, and cloned: the endothelial isoform (eNOS); the neuronal isoform (nNOS); and the inducible isoform (iNOS). All three isoforms are able to catalyze the production of NO from an assortment of precursors and co-factors, including L-arginine, NADPH, and oxygen. The different isoforms of NOS are found in a variety of cell types and tissues, including neuronal cells and the vascular endothelium. The localization of eNOS in the vascular endothelium is of particular importance for cardiovascular physiology, as eNOS maintains basal vascular tone through its release of low levels of NO.

NO has been extensively studied in the setting of myocardial I/R injury. Previous studies clearly demonstrate that the...
deficiency of eNOS exacerbates myocardial I/R injury\textsuperscript{12,13} whereas the overexpression of eNOS\textsuperscript{14,15} the administration of NO donors\textsuperscript{16,17} and inhaled NO gas therapy\textsuperscript{18} all significantly protect the myocardium.\textsuperscript{19} In terms of its cytoprotective mechanisms, NO possesses a number of physiological properties that makes it a potent cardioprotective-signalling molecule.\textsuperscript{20}\textsuperscript{26} First, NO is a potent vasodilator in the ischaemic myocardium,\textsuperscript{21} which allows for essential perfusion of injured tissue. Secondly, NO reversibly inhibits mitochondrial respiration.\textsuperscript{24} The inhibition of mitochondrial respiration during early reperfusion counterintuitively leads to a decrease in mitochondrial-driven injury by extending the zone of adequate tissue cellular oxygenation away from vessels.\textsuperscript{23} Thirdly, NO is a potent inhibitor of neutrophil adherence to vascular endothelium.\textsuperscript{24} Neutrophil adherence is an important event initiating further leukocyte activation and superoxide radical generation, which in turn leads to injury to the endothelium and perivascular myocardium.\textsuperscript{25} Fourth, NO also prevents platelet aggregation,\textsuperscript{26} which together with the anti-neutrophil actions of NO attenuates capillary plugging.\textsuperscript{27} Finally, NO inhibits apoptosis\textsuperscript{28} either directly or indirectly by inhibiting caspase-3-like activity via a cGMP-dependent mechanism and by direct inhibition of caspase-3-like activity through protein S-nitrosylation.\textsuperscript{29} While the majority of experimental studies investigating the effects of NO-based therapies in myocardial I/R have reported that NO protects the ischaemic myocardium, there have been some studies reporting negative effects of NO. A review by Bolli\textsuperscript{19} evaluated all of the studies investigating the role of NO in modulating the severity of I/R injury conducted between 1991 and 2001 in the non-preconditioned myocardium. This very through analysis of the literature revealed that 92 experimental studies had been conducted with 73% of all the studies concluding that NO (endogenous or exogenous) is protective while 20% reported no detectable effects, and 12% reported that NO was detrimental.\textsuperscript{19} A very important consideration when evaluating the efficacy of NO therapies in cardiovascular disease models is the concentration or dose of NO that is investigated. It is now well-appreciated that physiological levels (i.e. nanomolar) of NO promote cytoprotection and suprapharmacological levels (i.e. high micromolar and millimolar) mediate cellular necrosis and apoptosis.

A number of studies have evaluated the cytoprotective signalling pathways activated by NO in various experimental systems including isolated cardiac cells and intact hearts. NO has been shown to activate a number of cellular systems including isolated cardiac cells and intact hearts. NO has been shown to activate a number of cellular systems including isolated cardiac cells and intact hearts. NO has been shown to activate a number of cellular systems including isolated cardiac cells and intact hearts. NO has been shown to activate a number of cellular systems including isolated cardiac cells and intact hearts. NO has been shown to activate a number of cellular systems including isolated cardiac cells and intact hearts.
Nitrite has now moved to the forefront of NO biology with the discovery that it represents a critical NO storage form in both blood and tissues, which can be reduced to NO during ischaemic or hypoxic events (Figure 1). The liberated NO exerts biological effects at the level of the endothelium or diffuse into adjacent tissue and render effects through signalling mechanisms at the cellular level. This paradigm shift in nitrite physiology can be attributed to the findings of several key studies. The first set of studies from Zweier’s laboratory demonstrated that NO formation increased during myocardial ischaemia independent of enzymatic activity. Using electron paramagnetic resonance spectroscopy and chemiluminescence they observed that the generation and accumulation of NO from nitrite increases 100-fold under the acidic and highly reduced conditions of the ischaemic myocardium. The next set of studies demonstrated that the exogenous administration of nitrite could limit the extent of myocardial injury following ischaemia and reperfusion. Webb et al. first reported using a Langendorff isolated heart model that the infusion of nitrite (10 and 100 μM) prior to ischaemia reduced infarct size and was associated with comparable improvements in the recovery of left ventricular function. Duranski et al. reported that nitrite was cardioprotective in an in vivo mouse model of myocardial I/R injury. In this study, mice were subjected to 30 min of left coronary artery occlusion followed by 24 h of reperfusion. Sodium nitrite was administered at blood concentrations of 2.4–960 nM during myocardial ischaemia and was found to exert profound dose-dependent protective effects on cellular necrosis, with highly significant protective effects observed at near-physiological blood nitrite concentrations (48 nM). Consistent with hypoxia-dependent nitrite bioactivation, nitrite was reduced to NO, S-nitrosothiols, N-nitrosamines, and iron-nitrosylated haem proteins. Subsequent work has confirmed and expanded on these findings, demonstrating that nitrite therapy initiated at the onset of reperfusion protects the ischaemic myocardium and that nitrite therapy can also precondition the myocardium through either systemic or oral administration. Gonzalez et al. recently investigated whether a low dose of intravenous nitrite therapy would enhance the efficacy of reperfusion therapy for acute MI in a protocol compatible with typical delays from onset of chest pain to emergent intervention. In this study, they used an in vivo canine model with a protocol of 2 h of coronary artery ischaemia followed by 6 h of reperfusion. Nitrite therapy (0.20 μmol/min/kg) was administered either during the last 60 min of ischaemia or during the last 5 min of ischaemia. They found that nitrite therapy limited MI and apoptosis. Importantly, the mechanism of myocardial protection was found to be independent of the time/ischaemia severity integral because the group infused with nitrite during the last 5 min of ischaemia experienced a reduction in infarct size and apoptosis almost to a similar degree as the 60 min infusion group. This suggests that infusion of nitrite could be initiated prior to percutaneous coronary intervention.

Nitrite therapy has now been studied in a variety of in vitro and in vivo animal models and has proven to be an effective means to reduce I/R injury. However, there are two key questions regarding nitrite therapy that are still not answered. The first question is centred on how nitrite is reduced to NO during ischaemia. Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system, deoxyhaemoglobin, xanthine...
oxidase, and more recently myoglobin. The myoglobin angle is interesting, as it has long been considered a scavenger of NO. Secondly, the cytoprotective effects of nitrite therapy have not been fully elucidated. It has been universally shown that nitrite-mediated protection is independent of eNOS and haem oxygenase-1 enzyme activities and completely dependent on NO generation, as evidenced by the complete blockade of protection by the NO scavengers 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (carboxy-PTIO) and PTIO. Therefore, the cytoprotective effects of nitrite therapy are likely similar to those attributed to NO. Indeed, Shiva et al. have reported that the reduction of nitrite to NO results in the inhibition of complex I of the mitochondrial transport chain by post-translational S-nitrosation. This dampens electron transfer and effectively reduces ROS generation upon reperfusion and ameliorates oxidative inactivation of complexes II–IV and aconitase, thus preventing mitochondrial permeability transition pore opening and cytochrome c release.

4. Nitrite homeostasis and myocardial injury

Systemic nitrite levels are derived from both endogenous and exogenous sources. As much as 70% of plasma nitrite has been reported to originate from the oxidation of eNOS-derived NO, while the remaining 30% of plasma nitrite is derived from the consumption of nitrite and nitrate in dietary sources such as meat, vegetables (especially green leafy vegetables), and drinking water. In general, the dietary component of plasma nitrite is derived from nitrate, as humans do not generally consume significant amounts of nitrite in their diet. Nitrate enters the stomach and then circulates in the blood and is converted into nitrite by commensal bacteria in the mouth and gastrointestinal tract. Both sources of nitrite are important for maintaining steady-state nitrite levels in both the plasma and tissues, such as the heart (Figure 2). Recent data have shown that nitrite derived from either of these sources accumulates in the plasma and is stored in the heart and metabolized into NO during ischaemia.

Recent data have also shown that dysfunction of either source can result in an exacerbation of injury following myocardial ischaemia. For example, mice deficient in eNOS (eNOS) exhibit reduced steady-state levels of both plasma and cardiac nitrite levels when compared with wild-type mice, whereas, transgenic mice with a cardiac-specific overexpression of the human eNOS gene (eNOS-Tg) exhibit higher steady-state levels of both plasma and cardiac nitrite levels when compared with non-transgenic mice. Predictably, the deficiency of eNOS exacerbates myocardial I/R injury, whereas the overexpression of eNOS significantly protects the myocardium against I/R injury. Several studies have reported the benefits of dietary nitrite and nitrate as means to restore or enhance NO homeostasis. Bryan et al. recently investigated the effects of dietary nitrite and nitrate supplementation and insufficiency in mice on NO homeostasis and on the severity of myocardial I/R injury. In this study mice were fed a standard diet with or without nitrite (50 mg/L) supplemented in their drinking water for 7 days. Mice supplemented with nitrite exhibited significantly higher plasma levels of NOx. Nitrite homeostasis is determined by nitric oxide (NO) generation from NO synthases and dietary consumption of nitrate. Nitrate enters the stomach and then circulates in the blood and is converted into nitrite via salivary bacteria containing nitrate reductase. Nitrite derived from the diet and NOS activity rapidly accumulates in the plasma and is transported into tissues such as the heart. Nitrite is then stored in the myocardium and is metabolized into NO during hypoxia or ischaemia.
nitrite, significantly higher heart levels of nitrite and NO metabolites and displayed a 48% reduction in infarct size following myocardial ischaemia–reperfusion. Supplemental nitrate (1 g/L) in the drinking water for 7 days also increased blood and tissue NO products and significantly reduced infarct size. In addition, nitrite was consumed during the ischaemic phase with a concomitant increase in nitroso/nitrosyl products in the heart and plasma, which were restored to baseline levels by 30 min of reperfusion. In contrast, mice fed a diet deficient in nitrite and nitrate for 7 days exhibited significantly diminished plasma and heart levels of nitrite and NO metabolites and a 59% increase in infarct size following myocardial ischaemia–reperfusion. This data clearly show that dietary deficiency of nitrite and nitrite mimics the NO homeostasis found in eNOS−/− mice, suggesting that dietary sources of nitrite are just as important as endogenous sources of nitrite. This is supported by the findings of a follow-up study by this same group, which evaluated steady-state nitrite and NO metabolite levels in the plasma and hearts of eNOS−/− supplemented with nitrite (50 mg/L) in their drinking water for 7 days. They found that nitrite supplementation restored NO homeostasis in eNOS−/− mice and protected against myocardial I/R injury.

Collectively, these findings demonstrate the influence of eNOS-derived and dietary sources of nitrite on both plasma and myocardial levels of nitrite and NO homeostasis, as well as illustrate the cytoprotective effects of dietary nitrite supplementation and consequences of nitrite deficiency on the pathophysiology of myocardial I/R injury.

5. Nitrite and cardiovascular risk

In terms of nitrite homeostasis and cardiovascular risk, there is a small amount of indirect evidence to suggest that nitrite levels may serve as a predictor of risk for the development of cardiovascular disease. The endothelium plays an integral role in the regulation of vascular tone and blood pressure, platelet activity, leukocyte adhesion, and thrombosis and is intimately involved in the development of atherosclerosis. Primary cardiovascular risk factors such as hypertension, hypercholesterolaemia, and diabetes mellitus have all been shown to impair endothelial NO generation from eNOS and promote the severity of cardiovascular disease. Furthermore, coronary artery ischaemia and reperfusion has been shown to independently inhibit eNOS function via increased oxidative stress—an effect that results in pronounced deficiencies in myocardial NO levels. Therefore, the loss of NO generation as a result of a dysfunctional vascular endothelium could contribute to the development heart disease. Clinically, it has been shown that circulating levels of NO-related metabolites can serve as predictors of cardiovascular risk. A recent report by Kleinbongard et al. demonstrates that plasma nitrite levels progressively decrease with increasing cardiovascular risk. They evaluated intima media thickness and flow-mediated dilatation in patients with or without risk factors and cardiovascular disease and found that patients presenting with reduced flow-mediated dilatation and augmented intima to media thickness had lower levels of plasma nitrite compared with healthy individuals. However, with the exception of this one study, direct evidence is lacking on whether or not nitrite can serve as a reliable predictor for cardiovascular risk.

6. Evidence that nitrite is an endocrine molecule of nitric oxide

Given that NO possess diverse physiological actions and profound cytoprotective effects, it is important to understand its cell-signalling mechanisms. Classically, NO has been considered to be a paracrine-signalling molecule. This classification is based on several factors. First, NO has a very short (<2 ms) half-life in blood, owing to consumption by haemoglobin. Secondly, the half-life of NO within normoxic tissues has been estimated to be <0.1 s with diffusion distances dependent upon oxygen concentrations. Together, these two factors suggested that the transport of NO to distant sites was problematic. Thirdly, NO activity has been reported to be highly compartmentalized and intricately regulated. With the recognition that nitrite is a highly important, readily accessible storage form of NO that can be transported in the circulation, there has been a great deal of speculation that nitrite serves as an endocrine signalling molecule of NO. Early evidence that led to the speculation that nitrite serves as a NO transporter and facilitates remote physiological actions came from studies utilizing inhaled NO. In these studies inhaled NO has been shown to (i) increase urinary flow in pigs, (ii) decrease systemic vascular resistance in septic dogs, (iii) decrease systemic vascular resistance in anaesthetized sheep, (iv) increase intestinal blood flow during concurrent NO synthase inhibition or after intestinal I/R injury in cats, and (v) prevent forearm vasoconstriction during regional NO synthase inhibition in human volunteers.

While these studies clearly show that nitrite can act as an NO transporter and facilitate remote physiological actions, one could argue that these studies do not directly demonstrate that nitrite is an endocrine molecule of NO as widely speculated. The simple reason is that these studies used exogenous NO as the source of NO. Direct evidence that endogenous NO could act as an endocrine signalling molecule was not shown until recently when Elrod et al. reported that endogenously derived NO generated from eNOS is transported in the blood, metabolized in remote organs, and mediates cytoprotection in the setting of I/R injury. Utilizing a transgenic mouse with cardiac-restricted overexpression of eNOS, they found that the overexpression of eNOS in the myocardium resulted in increased levels of nitrite and other NO-metabolites in the circulation and subsequent transport and storage in liver. Importantly, increased tissue stores of nitrite in the liver attenuated hepatic I/R injury through formation of NO and nitroso/nitrosyl products. This report provides direct evidence that endogenously generated NO is capable of exerting endocrine activity via nitrite formation and therefore provides direct evidence supporting the reassessment of the perception that NO is merely a paracrine signalling molecule. It is important to note that the study by Elrod et al. also demonstrated similar increases in nitrosothiols in the eNOS transgenic mouse model and the authors suggest that
nitrosothiols may be responsible for the cardioprotective actions of NO.

7. Future research considerations

The field of nitrite biology has blossomed over the last decade. Nitrite is no longer considered to be an inert by-product of NO that is only useful as a marker for NO. Rather, it is now widely accepted and appreciated that nitrite is a physiologically relevant storage form of NO in blood and tissues that represents an alternate means for the generation of NO during certain pathological situations. Moving forward, the nitrite field faces some important questions that need to be addressed. First, other NO-related metabolites, such as nitrosothiols (RXNOs) have been shown to mirror nitrite in its effectiveness to promote cardioprotection and predict cardiovascular health.14,81–83 Heiss et al.107 reported that the plasma pool of RXNOs are depleted in patients with cardiovascular risk factors and S-nitrosothiols (RSNOs) have been shown to be protective in the setting of I/R injury.52,108 Furthermore, there is evidence to suggest that RSNOs may also serve as endocrine molecules of NO.81,109,110 So, the question then becomes, is nitrite or are RXNOs the main endogenous storage form of NO or are both equally important? Secondly, the means by which nitrite is transported from the circulation into tissues has not been identified. Thirdly, the primary nitrite reductase responsible for reducing nitrite to NO during myocardial ischaemia has not been identified. However, myoglobin has recently emerged as the main candidate. Although the primary function of myoglobin has been considered to be cellular oxygen storage and supply, recent studies have suggested classifying myoglobin as a multifactorial allosteric enzyme.79,111,112 In terms of NO biology, myoglobin has long been considered a scavenger of NO.79 Challenging this notion are two recent studies, which demonstrate that under conditions of hypoxia, myoglobin becomes the major nitrite reductase present in the myocardium.77,78 In these studies, it was demonstrated that the NO generated from nitrite by myoglobin escapes autocapture and regulates hypoxic mitochondrial respiration. Thus, the NO formed from nitrite reduction can inhibit respiring mitochondria to conserve tissue oxygen allowing for the diffusion of oxygen deeper into tissue (extension of oxygen gradients) and in the process protect the myocardium.78

There is no doubt that the field of nitrite biology and chemistry is an exciting area of research that continues to evolve. A direct result of our improved understanding of the biochemical conversion of nitrite to NO under both physiological and pathophysiological conditions has been a growing interest in the use of nitrite therapy to treat cardiovascular disease states. At present there are eight clinical studies underway (ClinicalTrials.gov) to investigate the potential vasculoprotective and cardioprotective actions of acute nitrite therapy in persons with cardiovascular risk factors or cardiovascular disease. Clinical trials are currently underway to evaluate the effects of nitrite therapy on pulmonary hypertension, preconditioning for ischaemic stress, vasodilator therapy, arterial hypertension, and sickle cell disease. The results of these ongoing clinical trials will provide very important insights into the potential clinical benefits of acute intravenous nitrite therapy as well as the effects of inhalation of sodium nitrite solutions. There are a number of advantages to using sodium nitrite over authentic NO gas or NO donor agents to treat cardiovascular diseases. Sodium nitrite is a highly stable compound that only releases NO under conditions of ischaemia, hypoxia, or low pH and therefore cardiovascular therapy with nitrite could be preferentially targeted to ischaemic or hypoxic tissues and reduce the risk of systemic hypotension. Furthermore, nitrite can be administered via inhalation, intravenous injection, intraperitoneal injection, and orally with NO only being released following the bioconversion of nitrite to NO in the circulation or ischaemic tissues. This is a tremendous advantage with sodium nitrite and is clearly not the case with NO gas or NO donors that spontaneously release NO and have limited routes of administration. It is conceivable that novel sodium nitrite formulations such as transdermal or subcutaneous preparations could be developed to promote local angiogenesis and wound healing in patients with impaired circulation such as diabetics. Finally, since sodium nitrite has been used for many years as part of the cyanide antidote kit in humans there is a wealth of clinical data to support the safety of very high doses of sodium nitrite in critically ill patients. No such data exists for NO gas and NO donors.

Given the very robust preclinical data regarding the cytoprotective effects of intravenous and dietary nitrite in I/R injury syndromes it is very logical to consider clinical trials of sodium nitrite therapy for acute MI, stroke, cerebral vasospasm, as well as an adjunctive therapy for transplantation of various organs. Given that oral administration of nitrite (i.e. dietary nitrite) mimics the cytoprotective actions of intravenous nitrite it would be possible to evaluate either acute intravenous nitrite or more chronic nitrite therapy following oral administration of sodium nitrite. Furthermore, since it has been reported that both oral and intraperitoneal administration of nitrite results in pharmacological preconditioning63 of the myocardium it would also be possible to administer nitrite 24 h prior to organ transplantation surgery, cardiac surgery, and balloon angioplasty procedures to precondition the patient against reperfusion injury.

A recent study by Kumar et al.113 is the first to demonstrate that chronic sodium nitrite therapy promotes vascular angiogenesis in a murine model system of hind-limb ischaemia. This study very clearly demonstrates that nitrite administered intravenously restores ischaemic hind-limb blood flow, stimulates endothelial cell proliferation, and stimulates angiogenesis in an NO-dependent manner. Injection of nitrite resulted in increased tissue nitrite levels, as well as nitrosothiol levels (SNO and XNO) in the ischaemic hind-limb. Furthermore, the pro-angiogenic actions of sodium nitrite were abrogated when an NO-scavenger agent (i.e. C-PTIO) was administered providing further evidence that these effects were largely NO-mediated. This study provides strong support that nitrite can undergo bioconversion to NO in ischaemic tissues and promote cell survival as well as vasculogenesis. The study by Kumar and coworkers provide...
a very strong impetus for the clinical investigation of sodium nitrite as a potential therapy for peripheral artery disease as well as therapy for chronic myocardial ischaemia and congestive heart failure. The future translation of nitrite from bench to bedside will certainly prove to be an exciting time in cardiovascular medicine and further insights gained from pre-clinical investigations will likely provide evidence for the investigation of various forms of sodium nitrite for other clinical conditions.

Despite the overwhelming enthusiasm for the clinical development of sodium nitrite for the treatment of cardiovascular disease it is very important to proceed with caution. There is a general agreement that very high levels of NO can promote tissue injury, decrease blood pressure, and cardiac performance all of which are contraindicated during any cardiovascular disease condition. Thus, it is extremely important to consider the timing of administration as well as the dosing when dealing with nitrite or any other agents that augment NO bioavailability. Finally, one must also consider the possible drug interactions between nitrite-based therapies and other agents to further avoid potential toxic side-effects.

Conflict of interest: D.L.J. is a participant of a pending U.S. patent filed on 14 October 2003 (patent no. 60:511244) regarding the use of sodium nitrite in cardiovascular disease. D.L.J. is a participant of a pending U.S. patent filed on 15 November 2007 (patent no. 61/003150) regarding the use of nitrite salts in chronic ischaemia.

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