Role of oxidised low density lipoprotein in atherogenesis

Joseph L Witztum

There can be little doubt that hypercholesterolaemia is a major risk factor for atherosclerosis. If plasma cholesterol concentrations rise above 160–180 mg/dl (4.1–4.7 mmol/l) the risk of developing clinical coronary artery disease (CAD) increases proportionately. Recent studies have convincingly demonstrated that lowering raised plasma cholesterol can reduce this risk and even induce regression. However, at any given concentration of plasma cholesterol there is still great variability in the expression of clinical CAD. This diversity undoubtedly reflects complex and multifactorial events that are involved in the reactions of the artery to hypercholesterolaemia and the resulting atherogenesis accompanied by alterations in coagulation and vasomotor tone that result in clinical events. The fact that subjects with familial hypercholesterolaemia, who have profound increases in low density lipoprotein (LDL) concentrations from birth, nevertheless show considerable diversity in the expression of clinical disease clearly shows that factors other than raised LDL concentrations are involved in the atherogenic process and the clinical sequelae.

Clearly many factors are involved in atherogenesis and it is likely that in any given individual one or more of these factors may be relevant, but I propose to review the evidence that modifications of LDL, and specifically oxidative modification of LDL, are critically important and possibly even prerequisites for macrophage uptake and accumulation of cellular cholesteryl ester in the artery wall. In this review I will discuss the following four points.

- What is oxidised LDL?
- What is the evidence for the presence in vivo of oxidised LDL?
- What are the potential mechanisms by which oxidised LDL may be atherogenic?
- What measures may be taken to inhibit the oxidative modification process?

I hope that this review will convince the reader that sufficient evidence has been accumulated to suggest that clinical trials in humans to test the antioxidant process are currently indicated.

What is oxidised LDL?

The concept that modification of LDL is a prerequisite for macrophage uptake and cellular accumulation of cholesterol has been reviewed in detail.^{1,2} Here I will summarise

the evidence that has led to this conclusion. With the discovery by Brown and Goldstein of the LDL receptor pathway, it seemed reasonable at first to suppose that this would be the mechanism by which LDL was taken up by macrophages—the cell type that chiefly accounts for the “foam cells” that typify the fatty streak. However, LDL incubated with macrophages in culture failed to have sufficient uptake to cause cholesteryl ester accumulation and furthermore, subjects (or rabbits) completely lacking LDL receptors showed the most advanced atherosclerosis. Therefore, another mechanism had to be invoked. Again, it was Goldstein *et al* who first proposed that modification of LDL was a prerequisite for macrophage uptake and cholesterol accumulation.³ They demonstrated that a chemical derivatisation of LDL, acetylation, led to enhanced macrophage uptake by a novel receptor, termed the scavenger receptor, and led to cholesteryl ester accumulation. Other chemically modified forms of LDL, such as malondialdehyde-conjugated LDL (MDA-LDL) were also recognised by the same receptor. Subsequent studies by Steinberg and colleagues showed that incubation of LDL with cultured endothelial cells, or smooth muscle cells, converted LDL into a modified form now taken up more rapidly by macrophages. It was Steinbrecher *et al*⁴ who then showed that this was due to the ability of these cells to initiate lipid peroxidation in the LDL, a finding confirmed for smooth muscle cell modification as well.⁵ Important parallel studies showed that LDL was cytotoxic to endothelial cells and smooth muscle cells and that the cytotoxicity was caused by oxidation of LDL lipids occurring during the incubation.⁶ It is now clear that oxidation of LDL can be induced by incubating it under appropriate culture conditions with various cells, including endothelial cells, smooth muscle cells, macrophages, or even fibroblasts.

Oxidation can also be induced by incubation of LDL with a known catalyst of lipid peroxidation, such as copper or iron.⁴ The exact mechanisms by which cells initiate lipid peroxidation of LDL in the culture medium have not been fully defined. It seems that several mechanisms may operate. For example, cellular lipoxygenases, such as 15-lipoxygenase (15-LO), are likely to affect the ability of endothelial cells and macrophages to induce such modification,⁷ and superoxide anion may be secreted into the medium by cells such as smooth muscle cells.^{5,8} Once such an LDL is

Department of
Medicine, University
of California, San
Diego, La Jolla,
California, USA

J L Witztum

Correspondence to
Dr Joseph L Witztum,
Department of Medicine,
0682, University of
California,
San Diego, La Jolla,
CA 92093-0682, USA.

Role of oxidised LDL in atherosclerosis and methods of detecting the changes at various stages of the process. (Reproduced with permission from the *Journal of Clinical Investigation*.²)

“seeded” with lipoperoxides, copper ion present in the medium will generate peroxy radicals, leading to chain reactions which result in amplified numbers of lipoperoxides, rearrangement of fatty acid double bonds, and the resulting characteristic formation of “conjugated dienes”⁹ (see figure). In vitro such oxidative modification of LDL is dependent on the presence of copper (or iron) ions and is completely inhibited by metal chelators. Furthermore, during the oxidative modification there is extensive conversion of lecithin to lysolecithin, catalysed by a phospholipase A_2 activity intrinsic in the LDL.¹⁰ Presumably, this results in the release of oxidised fatty acids which can further promote the propagation reaction. Consequent to this propagation, fatty acid fragmentation occurs, leading to the formation of highly reactive intermediates, such as aldehydes and ketones, and other oxidised products, such as oxysterol derivatives. In part, these reactive intermediates may then complex with the adjacent apo B protein, as well as with phospholipids. The modification of the lysine

residues (and other amino acids as well) of apo B inhibits the ability of LDL to bind to the LDL receptor, and when sufficient protein modification has occurred, this results in the generation of new epitopes on apo B that are now recognised by macrophage scavenger receptors.^{11,12} Freubis *et al* proposed that reactive fatty acid fragments may directly bind to apo B protein residues without the need for intermediate reactive aldehydes.¹³ Though the figure suggests an ordered series of reactions, in fact the complex reactions occur in a variable and even chaotic manner.

Consequently, it should be clear that oxidised LDL is not a single, homogeneous entity, but it is a particle containing heterogeneous products that include oxidised fatty acids and their breakdown products, oxidised sterols, oxidised phospholipids, as well as adducts of these various lipid products with apo B and phospholipids. In addition, some of these products may become quite polar and thus able to leave the LDL particle and mediate one or more of the biological effects that oxidised LDL has been reported to produce. In practical terms, it should be realised that oxidised LDL particles are quite heterogeneous, and furthermore, that the products formed from preparation to preparation may vary even when efforts are made to hold conditions of oxidation constant. These differences may depend on the cell types used to initiate oxidation, the metal ion concentrations, composition of the medium, the experimental conditions, and probably most importantly, the differences in inherent susceptibility to modification of different LDL preparations. A consequence of this is that various different effects of oxidised LDL on biological systems have been described, at times giving conflicting results between different laboratories. Such variability may be due in part to the fact that different components of the oxidised LDL may have different biological effects. This is discussed in further detail in our recent review,² but this should serve to add a note of caution to the reader in interpreting the myriad of effects that oxidised LDL has now been reported to produce (see below).

We have assumed that the oxidative modification of LDL occurs primarily in the artery wall, in the intima, and most probably in microdomains sequestered from the many plasma antioxidants. It seems unlikely that significant degrees of oxidative modification of LDL can occur in plasma because of the many antioxidants present. Furthermore, were sufficient oxidation of LDL to occur in plasma, that oxidised LDL would be removed rapidly from plasma by scavenger receptors present on hepatic sinusoidal cells. However, plasma LDL could undergo limited oxidation which though it had no consequences for the LDL within the vascular space, might nevertheless lead to an LDL “primed” for more rapid oxidative modification in the intima. It seems likely that LDL is modified when LDL is on the surface of cells, such as endothelial cells or macrophages, or while bound to inti-

Table 1 Evidence for a role of oxidised LDL in atherogenesis

- (1) Epidemiological evidence showing an inverse relation between dietary/plasma antioxidants and CAD.
- (2) Immunocytochemical evidence of oxidised LDL in atherosclerotic lesions.
- (3) Evidence that LDL extracted from lesions has physiochemical, immunological, and biological properties of oxidised LDL.
- (4) Presence in serum of autoantibodies with specificity for epitopes of oxidised LDL.
- (5) Presence in lesions of IgG with specificity for epitopes of oxidised LDL.
- (6) Presence in serum of subfractions of LDL with properties similar to early stages of oxidised LDL.
- (7) Ability of antioxidants to inhibit atherosclerosis in animal models.

Table 2 Potential mechanisms by which oxidised LDL has an impact on the atherogenic process

- (1) By promoting macrophage cholesteryl-ester enrichment through enhanced uptake through one or more scavenger receptors.
- (2) By releasing products that are chemotactic for monocytes and T lymphocytes.
- (3) By inhibiting the ability of macrophages to egress from the artery.
- (4) By releasing products that are cytotoxic.
- (5) By altering gene expression of arterial cells, such as inducing leucocyte adhesion molecules on monocytes and inducing synthesis of MCP-1 or IL-1.
- (6) By being immunogenic and potentially inducing humoral or cell mediated immunological responses.
- (7) By adversely affecting coagulation pathways, as for example by induction of tissue factor synthesis by macrophages.
- (8) By adversely affecting vasomotor properties of coronary arteries.

mal matrix, possibly in micro-sequestered loci created by extensions of cells, as demonstrated for macrophages by Heiple and colleagues.¹⁴

The implications of this argument are that it is primarily the antioxidant content of the LDL itself that is critical for its protection, and that the antioxidant environment of the intima and the ability of arterial cells to initiate lipid peroxidation may be the critical variables in determining the extent of arterial wall modification of LDL.

Evidence of the presence in vivo of oxidised LDL

Steinberg and I have reviewed in detail the evidence that oxidised LDL is present in vivo.² Table 1 summarises the different lines of evidence that support this contention. First, epidemiological evidence of an inverse relation between dietary and/or plasma concentrations of antioxidants and the development of CAD is accumulating. Thus there is evidence that either levels of dietary intake or plasma concentrations or both of vitamin E, vitamin C and β -carotene are inversely related to CAD.¹⁵⁻¹⁸ Furthermore, countries where consumption of monounsaturated fatty acids is high, with consequent enrichment of LDL with these fatty acids and more resistance to oxidative modification in general have lower rates of CAD. Second, immunocytochemical evidence, gained with antibodies to various epitopes on oxidised LDL showed intense staining of atherosclerotic lesions in both animals and humans but not of normal arterial tissue.¹⁹⁻²¹ Third, LDL extracted from lesions has been shown to have many of the physiochemical, immunological and, most importantly, biological properties attributed to in vitro preparations of oxidised LDL.²² Fourth, oxidised LDL is immunogenic and the presence in serum of animals and humans of autoantibodies with specificity for the epitopes of oxidised LDL has been demonstrated.²⁰ In particular, a recent study of eastern Finnish men found that the titre of antibodies to an epitope of oxidised LDL was highly predictive of the subsequent progression of carotid artery atherosclerosis, implicating the oxidative process in atherogenesis.²³ Fifth, in atherosclerotic lesions IgG with specificity for epitopes of oxidised LDL can be demonstrated, again suggesting the presence of oxidised LDL. Sixth, whereas extensive forms of oxidised LDL are not found in serum, several investigators have reported that plasma may contain small numbers of LDL particles that have the properties of LDL that has undergone the early steps of oxidative modification.

Though all of the above lines of evidence represent qualitative evidence that oxidised LDL is present in the artery wall, they do not provide information as to the quantitative importance of this process. However, if oxidation is important, then inhibiting it should inhibit the atherogenic process, and indeed, there are now numerous studies showing that

lipophilic antioxidants, that are carried in the LDL core and which protect LDL as measured by in vitro assays, inhibit atherosclerosis in studies of hypercholesterolaemic rabbits and primates. Six different studies that used three different lipophilic antioxidants in hypercholesterolaemic rabbits showed inhibition of atherosclerosis ranging from 30 to 70%.²⁴⁻²⁹ Recently, probucol has been shown to inhibit atherosclerosis in primates as well, independently of its ability to lower LDL concentrations.³⁰ Furthermore, these effects could be shown to be independent of alterations in plasma LDL concentrations. The pioneering studies of Carew *et al*²⁴ clearly demonstrated that antioxidant therapy with probucol inhibited the uptake and degradation of LDL in portions of aorta containing atherosclerotic lesions, but not in portions of normal arteries. This is consistent with a direct effect of the antioxidants to prevent oxidative modification in macrophage-rich lesions. While it is conceivable that these antioxidants are inhibiting atherogenesis by as yet undefined mechanisms unrelated to their ability to protect LDL, it seems more likely that it is indeed their ability to protect LDL from oxidation that accounts for their ability to inhibit atherogenesis. Thus there are numerous lines of evidence strongly supporting the hypothesis that oxidative modification of LDL is an important factor, if not prerequisite, for mediating the atherogenicity of LDL.

Potential mechanisms by which oxidised LDL may have an impact on the atherogenic process (table 2)

Interest in the role of oxidised LDL began with the realisation that oxidised LDL had enhanced uptake in macrophages and promoted cholesteryl ester accumulation. However, it is now apparent that many other diverse biological effects may be induced by oxidised LDL.^{1,2} As noted above, many different products are generated during the oxidative modification of LDL and presumably each of these may have diverse biological effects. Thus products of oxidised LDL have been reported to be chemotactic for monocytes, which are widely regarded as the precursors of most of the foam cells that form in the fatty streak. Potentially of equal importance is the recent realisation that up to $\geq 20\%$ of the early cells found in the fatty streak are T lymphocytes. Recent studies have suggested that the products of oxidised LDL are also chemotactic for this subclass of lymphocytes as well. A third potential mechanism is the fact that oxidised LDL inhibits migration of macrophages and thereby prevents their egress from the artery wall. One would ordinarily presume that once macrophages had picked up toxic products, such as oxidised LDL, they would move out of the area and thereby restore it to normal. The fact that macrophages take up oxidised LDL but then remain may be an important event in the pathological accumulation of

cholesterol in the artery wall. This may be worsened by the fact that products of oxidised LDL are highly cytotoxic to various cells, including endothelial cells. Disruption of endothelial cells after the formation of an intimal fatty streak has been demonstrated in experimental models, and the subsequent exposure to circulating blood elements, such as platelets, may be vital to the subsequent introduction into the lesion of growth factors, such as PDGF (platelet derived growth factor) that initiate the migration and proliferation of smooth muscle cells, which then convert the fatty streak into the more complicated lesion. Furthermore, the toxic effects of oxidised LDL, either from extracellular oxidised LDL in the intima, or possibly even from products within the macrophages filled with oxidised LDL, may initiate the cell death of macrophages, which typifies the more advanced lesion, leading to the frank deposition of "ceroid", the insoluble lipid that makes the gruel of the advanced lesion. Studies from my laboratory and others³¹ have shown that a component of ceroid is derived from oxidised LDL.

When LDL first enters the intimal space and is exposed to pro-oxidant conditions it undergoes the earliest steps of the oxidative supplement modification, and numerous products may be generated even though the LDL is not yet sufficiently modified so as to be recognised by the macrophage scavenger receptor. This so-called "minimally oxidised" or "minimally modified" LDL nevertheless may have an important role in the atherogenic process by releasing products that affect the gene expression in arterial cells. For example, such minimally oxidised LDL can stimulate the expression and secretion of M-CSF, GM-CSF, and G-CSF in human aortic endothelial cells,³² and when injected in vivo in mice, caused a 7–26 fold increase in serum M-CSF activity.³³ Such minimally oxidised LDL can stimulate monocyte-specific adherence molecules on the surface of endothelial cells, thereby leading to yet another mechanism for monocyte chemoattraction, and, importantly, can stimulate the secretion of monocyte chemotactic protein-1 (MCP-1) by cultured human aortic, endothelial, and smooth muscle cells³⁴ and when injected in vivo in mice can increase the mRNA expression of JE (the mouse homologue of MCP-1) in liver and other tissues.³³ Using in situ hybridisation techniques, we have demonstrated that MCP-1 and M-CSF message are found in macrophage-rich regions of both rabbit and human aortas but not in normal aortas.^{35,36} Thus it is likely that oxidised LDL can induce arterial wall cells to produce various potent chemotactic factors, adhesion molecules, cytokines, and growth factors that have an important role in development and maturation of the atherosclerotic plaque. Though we do not know at present which of the many different products generated during the oxidative modification of LDL are responsible for these different effects, this is an exciting area for future research that

undoubtedly will have important long-term consequences.

We have previously shown that even minor modifications of LDL are immunogenic and that oxidised LDL is immunogenic too. Autoantibodies against various epitopes of oxidised LDL are present in human serum, and immunoglobulin specific for epitopes of oxidised LDL can be found in atherosclerotic lesions. Whether or not these antibodies are mere epiphenomena reflecting an immune response to altered proteins and lipids is at present unknown, but it is of interest that the titre of autoantibody to an epitope of oxidised LDL, MDA-lysine, was an effective predictor of the progression of carotid atherosclerosis in a recent study.²³ Since many lines of evidence suggest that there is activation of immune cells within the artery wall, the presence of specific immune complexes could provide at least one mechanism by which activation of T cells could occur, resulting in cell-mediated immunological responses. Since T cells are clearly an important component of the early fatty streak lesion, this is an area that we need to know much more about (reviewed in reference 37).

Finally, oxidised LDL may alter two vital properties that result in the often fatal sequelae that mark the atherosclerotic artery. As outlined elsewhere in this supplement, it is clear that thrombotic events can occur on lesions that are only modestly involved with atherosclerotic events and that plaque rupture and initiation of thrombosis can occur at the lateral margins of such lesions. In this regard, the observation that macrophages containing oxidised LDL are also found in such regions is of potential importance, particularly in light of the recent report that oxidised LDL may stimulate the release of tissue factor by macrophages.³⁸ In addition, there is now an extensive body of information suggesting that hypercholesterolaemia in itself may impair the EDRF-mediated vasorelaxation of coronary arteries in response to agents, such as acetylcholine. In large part, this inhibition seems to be mediated via products of oxidised LDL, either through direct disruption of receptor-mediated signalling to the nitric oxide synthase, or by interfering with the nitric oxide (or nitric oxide/thiol adduct) that is the EDRF factor (see reference 39). Thus hypercholesterolaemia in itself, by generating more oxidised LDL in the arterial intima, may alter vasomotor responses, even producing paradoxical vasoconstriction in response to normal vasodilatory stimuli, and contribute importantly to vasospasm, even in the absence of significant lesions. Of course, this may be greatly accentuated in those areas where fatty streaks or intermediate lesions are present. Thus, lowering cholesterol and inhibiting oxidation may have profound effects on the clinical events marking CAD, even without causing major changes in arterial wall structure.

In summary, while it remains to be proved that all or even some of these phenomena have in vivo counterparts, the concept is

clearly emerging that oxidised LDL may contribute importantly to the atherogenic process via mechanisms above and beyond that of contributing to cholesterol deposition in the plaque and narrowing of the vessel lumen. In fact, it is possible that oxidised LDL may promote the atherogenic process by virtue of its oxidation products, even before or coincident with the delivery of lipid to the arterial cells. This implies that factors that inhibit the oxidation of LDL may have even more profound effects on inhibiting the atherogenic process and the resulting clinical events than can be measured simply by inhibition of plaque formation.

Measures to inhibit oxidative modification of LDL

One can approach the subject of inhibition of the oxidation of LDL *in vivo* by understanding those factors that lead to this process. First, we must consider factors intrinsic to the LDL particle itself. The nature of the substrate for lipid peroxidation, mainly the polyunsaturated fatty acids in lipid esters and cholesterol, is a dominant influence in determining susceptibility. As noted by Esterbauer *et al* there is a vast excess of polyunsaturated fatty acids in LDL, chiefly in the form of linoleic acid, in relation to the content of natural, endogenous antioxidants.⁹ The importance of the fatty acid composition has been demonstrated by recent studies in our laboratory in rabbits and in humans that showed that increasing the oleic acid content of LDL, at the expense of linoleic acid, will greatly protect the LDL from oxidation.⁴⁰⁻⁴² Secondly, the endogenous content of natural antioxidants, including vitamin E, β -carotene, ubiquinol-10, and others (as well as the presence of exogenously administered antioxidants such as probucol) are exceedingly important. Studies by Esterbauer *et al* clearly show that these endogenous antioxidants must be consumed before the initiation of the rapid propagation phase of lipid peroxidation.⁹ A third factor that seems to affect the susceptibility of LDL to oxidation is particle size—with smaller, more dense LDL subfractions displaying an apparent increased propensity to be oxidised. Theoretically, differences in the intrinsic phospholipase A₂ activity, which greatly promotes propagation reactions, may also be an important determinant.

In addition to the inherent susceptibility of the given LDL particle, factors extrinsic to LDL that promote oxidation also may be vitally important. Such factors may include potential variation in cellular pro-oxidant activity, as for example differences in the ability of macrophages to modify LDL because of inherent differences in macrophage expression of 15-lipoxygenase or the ability of cells to secrete cellular superoxide anion or thiols.^{43,44} Secondly, the concentration of plasma and extracellular fluid peroxidant components, such as trace metal concentrations, may be involved, as is the

concentration of binding proteins that complex with these metals. A third possibility is the concentration of plasma and extracellular fluid antioxidant compounds such as urate and importantly, ascorbate. Though ascorbate is hydrophilic, nevertheless it is capable of converting the alpha tocopherol radical (generated when vitamin E acts as an antioxidant) to reduced alpha tocopherol, thereby recycling it to act again as an antioxidant.^{9,45} Presumably, at the interface between an LDL particle and ascorbate, such a reaction could help to maintain an effective vitamin E concentration in an LDL particle. HDL has also been shown to inhibit the oxidation of LDL in cell culture⁴⁶ and in a model system,⁴⁷ though the mechanisms for this property remain unknown. Finally, factors influencing the residence time of LDL in the intima are undoubtedly very important as well—that is, factors that increase binding of LDL such as Lp(a), differences in localised matrix proteins and glycoproteins that bind LDL, or processes such as non-enzymatic glycosylation of LDL or matrix, all of which could prolong the residence of LDL in the intima and thereby increase the potential for it to be oxidised.

From the discussion above it follows that interventions to inhibit oxidative modification can be thought of as being focused in these two general areas: measures to protect LDL itself and measures to reduce those factors responsible for initiating oxidation of LDL. At present, probucol is the most potent lipophilic antioxidant capable of protecting LDL. Its demonstrated ability to inhibit atherosclerosis in rabbits, despite its ability to lower HDL, suggests that it is a prime candidate to use in testing the antioxidant hypothesis in humans. Recent data from our laboratory in which oleic acid enrichment of LDL was achieved at the expense of linoleic acid shows the principle of this type of dietary intervention to inhibit the oxidative process,⁴⁰⁻⁴² but the extent to which such protection can be achieved in free-living populations remains to be fully demonstrated. Though the studies used extreme changes in diet, it nevertheless seems reasonable to believe that moderate increases in monounsaturated fatty acids, at the expense of polyunsaturated fatty acids, while producing moderate changes, may have important clinical effects when integrated over long periods.

Supplementation with natural lipophilic antioxidants, such as vitamin E and β -carotene, also seems appropriate. Indeed, in studies in which human subjects were supplemented with vitamin E it was shown that the vitamin E content of LDL was enriched two to three fold, and these LDLs were significantly less susceptible to oxidative stress.⁹ Theoretically β -carotene should produce a similar effect, but there are no data, at least *in vivo*, to show that β -carotene feeding will protect LDL. It must be recognised that antioxidants, such as probucol or vitamin E, may also have important effects in inhibiting the ability of cells to modify LDL, as recently demonstrated by Parthasarathy using a water-

soluble form of probucol.⁴⁸ While dietary supplementation with the natural antioxidant vitamins, such as E and β -carotene (and presumably other carotenoids) seems reasonable, particularly because there are no concerns about the side effects, as yet there are no prospective data on their effectiveness in vivo. Combinations of these interventions could prove synergistic, like the dietary supplementation of ascorbic acid noted above. Such interventions are currently feasible and intensive study is now underway to determine the optimal forms of intervention to protect LDL. A recent consensus conference supported the notion that sufficient evidence was available to encourage intervention trials in humans to test this hypothesis.⁴⁹ It seems reasonable to use probucol and a vitamin cocktail (for example, containing vitamin E, vitamin C and β -carotene) or both to test the antioxidant hypothesis in humans.

On the other hand experimental approaches designed to inhibit the ability of cells to modify LDL are more theoretical at present, but may offer alternative approaches as well. For example, if it can be demonstrated that inhibition of macrophage 15-lipoxygenase activity in animal models inhibits atherosclerosis, then this would offer a novel approach that would act synergistically with measures directly to protect the LDL itself. Finally, it should be noted that perhaps the most effective way to inhibit the oxidative modification of LDL is to reduce plasma LDL concentrations, thereby reducing the substrate in a most direct manner.

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