Measurement of Copper-Binding Sites on Low Density Lipoprotein

Alexander Roland, Rebecca A. Patterson, David S. Leake

Abstract—Copper is often used to oxidize low density lipoprotein (LDL) in experiments in vitro and is a candidate for oxidizing LDL in atherosclerotic lesions. The binding of copper ions to LDL is usually thought to be a prerequisite for LDL oxidation by copper, although estimates of LDL copper binding vary widely. We have developed and validated an equilibrium dialysis assay in a MOPS-buffered system to measure copper binding to LDL and have found 38.6±0.7 (mean±SEM, n=25) copper binding sites on LDL. The binding was saturated at a copper concentration of 10 μmol/L at LDL concentrations of up to 1 mg protein/mL. Copper-binding capacity increased progressively and markedly when LDL was oxidized to increasing extents. Chemical modification of histidyl and lysyl residues on apolipoprotein B-100 reduced the number of binding sites by 56% and 23%, respectively. As an example of the potential of this method to assess the effects of antioxidants on copper binding to LDL, we have shown that the flavonoids myricetin, quercetin, and catechin (but not epicatechin, kaempferol, or morin), at concentrations equimolar to the copper present (10 μmol/L), significantly decreased copper binding to LDL by 82%, 56%, and 20%, respectively. (Arterioscler Thromb Vasc Biol. 2001;21:594-602.)

Key Words: low density lipoproteins ■ atherosclerosis ■ copper ■ flavonoids ■ quercetin

The oxidation of LDL is widely believed to be an important event in the pathogenesis of atherosclerosis.1,2 Oxidized LDL may be endocytosed in an uncontrolled manner by macrophages, resulting in the generation of cholesterol-laden foam cells, which characterize atherosclerotic lesions.1,2 In addition, oxidized LDL is chemotactic for leukocytes,3 is cytotoxic,4 can induce smooth muscle cell proliferation,5 and has many other potentially atherogenic effects.

The presence of active transition metal ions is usually a prerequisite for LDL oxidation by cells, at least in vitro.6–10 and LDL can also be oxidized by transition metal ions in the absence of cells.6–8 The mechanisms of LDL oxidation in atherosclerotic lesions are unclear, but transition metal ions may be involved.9 Catalytically active copper and iron have been reported to be present in atherosclerotic lesions,12–14 and the copper-carrying protein of plasma, ceruloplasmin, can catalyze LDL oxidation.15,16 At pH 7.4, only the single most loosely held copper ion on ceruloplasmin appears to be required to catalyze LDL oxidation,15 although the other copper ions on ceruloplasmin may be important at acidic pH.16,17 In addition, there appears to be a trend toward increased levels of α- and m-tyrosine in human advanced atherosclerotic lesions, which may be indicative of the presence of redox-active copper.18 However, high levels of 3-nitrotyrosine, indicative of the presence of the oxidizing agent peroxynitrite, can also be found in LDL isolated from atherosclerotic lesions.19 Evidence of the importance of the pro-oxidant enzyme myeloperoxidase can be found in early as well as in advanced atherosclerotic lesions.20 These mechanisms and others may play a role in atherosclerosis, although their relative importance may differ at different stages of the disease.

For in vitro studies of oxidized LDL, oxidation by copper is frequently used and produces LDL with characteristics similar to LDL oxidized by cells.1 Copper is more potent than iron in its ability to oxidize LDL in vitro.21 The oxidation of LDL by copper is likely to require the binding of copper ions to the lipoprotein particle. This binding occurs, at least in part, to histidine-containing sites on apoB-100, the protein moiety of LDL.22,23 The number of binding sites reported varies widely over 2 orders of magnitude22–33 (Table).

A number of antioxidants may act in part by inhibiting the binding of copper (or iron) to LDL. Flavonoids provide a potentially important example of antioxidants that may function in this way. Flavonoids are potent inhibitors of LDL oxidation by transition metal ions and cells,34–36 and dietary intake of these compounds has been inversely correlated with cardiovascular disease in several, but not all, epidemiological studies.37–43 Most biochemical studies have focused on the in vitro free radical–scavenging activity of flavonoids, but a number of flavonoids are capable of forming complexes with transition metal ions.44–47

In the present study, we report an equilibrium dialysis assay that has allowed us to determine the number of binding sites for copper on LDL, and we demonstrate its value in assessing the
ability of antioxidants for LDL to inhibit the binding of copper to these lipoproteins by the use of flavonoids.

Methods

LDL Isolation and Oxidation

LDL (density 1.019 to 1.063 g/mL) was isolated from the blood of healthy volunteers by sequential density ultracentrifugation at 4°C in KBr solutions, as described elsewhere. Oxidation was carried out to defined extents at 37°C in Dulbecco’s PBS as previously described. Briefly, LDL (100 μg protein/mL) was incubated with CuSO4 (Fisher Scientific UK) at a concentration 5 μmol/L above that of the EDTA carried over from the LDL preparation. Conjugated diene formation was monitored at 234 nm. Oxidation was stopped by the addition of EDTA (1 g/L). Mildly oxidized LDL was defined as LDL that had demonstrated an absorbance increase of 0.2, moderately oxidized LDL was defined as LDL in which peak absorbance had been reached (after ~3 hours), and highly oxidized LDL was defined as LDL whose oxidation had been terminated after 24 hours. The density of each sample was adjusted to 1.2 g/mL with solid KBr, in the presence of washed Chelex-100 chelating resin (Sigma). Chelex was prewashed in distilled water to remove any contaminating antioxidant activity associated with the beads. After centrifugation at 250g for 10 minutes to sediment the Chelex, LDL was ultracentrifuged at 150 000g for 18 hours at 4°C. The oxidized LDLs were then dialyzed against PBS, pH 7.4, consisting of NaCl (140 mmol/L), Na2HPO4 (8.1 mmol/L), NaH2PO4 (1.9 mmol/L), and EDTA (100 μmol/L), before filter sterilization. LDL was stored in darkness under argon at 4°C and was used within 4 weeks. Ethics committee approval was obtained for the isolation of LDL at the University of Reading, and blood donors gave their informed consent.

Copper Binding to LDL

Buffers were prepared at 4°C and pretreated with washed Chelex-100 (1 g/L) to remove traces of transition metals. LDL was extensively dialyzed in dialysis tubing (10-mm flat width) with a molecular weight cutoff of 12 to 14 kDa (Medicell International Ltd) against the phosphate buffer described above but without EDTA for at least 12 hours at 4°C, with 3 buffer changes to remove EDTA. A further buffer exchange was then carried out by dialyzing at 4°C against a MOPS buffer, pH 7.4, consisting of NaCl (150 mmol/L), MOPS (10 mmol/L), and butylated hydroxytoluene (BHT, 20 μmol/L), pH 7.4 (MOPS and BHT were obtained from Sigma). The LDL samples were then diluted to 1 mg LDL protein/mL by using MOPS buffer containing CuSO4 (10 μmol/L added from a 10-mm stock solution in water), with or without flavonoids (10 μmol/L), and 1 mL was loaded into Medicell dialysis tubing or 15-kDa cutoff Spectra/Por 2.1 high-speed dialysis tubing (Spectrum). Copper binding to LDL was carried out in 500 mL MOPS buffer containing copper and flavonoids at 4°C, pH 7.4, until saturation of binding was achieved (24 hours with Medicell tubing, 10 hours with Spectra/Por 2.1 tubing). Gentle stirring of buffer solutions was carried out at each dialysis stage to ensure mixing without inducing LDL aggregation. After dialysis, LDL protein was measured by use of a modified Lowry assay, after the LDL was diluted 10-fold in water. An equivalent volume of MOPS buffer was included in the BSA standards, because MOPS increased the absorbances obtained in the protein assay. For experiments with other buffers, the above procedure was carried out exactly as described above but substituting MOPS with 10 mmol/L of one of the following buffers: MES, HEPES, phosphate, or Tris. NaCl (0.15 mol/L) of “pH 7.4” was also tested. When alternative buffers were tested, these were also included in the BSA standards of the protein assay.

Quantification of Copper

Copper ions were measured spectrophotometrically by use of the indicator molecule bathocuprocinnid sulfonic acid (BC, Sigma), the Cu(II) complex of which absorbs strongly at 480 nm. Triplicate samples of LDL were diluted 4-fold in MOPS buffer; control samples without LDL were obtained from the bulk dialysis solution.
and from control dialysis bags containing only copper and dialysis buffer to confirm that the “free” copper concentrations were equal on both sides of the dialysis membrane. A 0.6-ml volume of each diluted sample was added to 50 µL BC stock solution in water (to give a final concentration of 400 µmol/L BC), together with 50 µL ascorbate (sodium salt, Sigma) stock solution freshly prepared in water (to give a final concentration of 1 mmol/L ascorbic acid). The mixture was incubated at room temperature for 5 minutes, during which any Cu(II) was reduced to Cu(I), resulting in BC-Cu(I) complex formation. A further 0.6-ml sample of each diluted LDL solution was incubated with EDTA (1 ml added in a 100-µL volume of stock EDTA in water), for the reason explained in Results. Absorbances were measured at 480 nm, and the values obtained in the presence of EDTA were deducted from the values obtained in the presence of BC/ascorbic acid (the former values were typically 10% to 20% of the latter). Copper levels were determined by comparison with a CuSO₄ standard plot up to 100 µmol/L.

Calculation of Copper Ions Bound per LDL Particle

To determine the number of copper ions associated with each lipoprotein particle, LDL concentrations were converted to micromolar units (a value of 513 kDa was used for the molecular mass of apoB-100). After deducting the free copper concentration (see above) from the total concentration of copper associated with the LDL, the concentration of LDL-associated copper was divided by the concentration of LDL to give a ratio of copper ions bound per LDL particle.

We examined whether the presence of LDL interfered with the standard plot for the copper assay. Standard plots (0 to 100 µmol/L copper) in the presence of LDL (250 µg protein/mL before addition of EDTA or BC and ascorbate), obtained by deducting EDTA-corrected absorbances from those in the presence of BC and ascorbate, revealed that such plots were indistinguishable from those obtained in the absence of LDL at copper concentrations <80 µmol/L (data not shown). Because copper concentrations in the LDL-containing dialysis bags measured after dilution were ~20 µmol/L, we consider the correction method using EDTA to be acceptable. The EDTA correction method allows for the endogenous absorbance of LDL at 480 nm and the very low level of aggregation of LDL induced by copper during the time scale of the assay (see Results).

Chemical Modification of ApoB-100

Histidyl residues were modified by incubation with diethylpyrocarbonate, as described by Chen and Frei. Briefly, LDL (100 µg protein/mL) was incubated with diethylpyrocarbonate (1 mmol/L) in PBS for 10 minutes at 37°C. The progress of the reaction and the amount of modification were monitored spectrophotometrically at 240 nm. After histidyl modification, samples were adjusted to a density of 1.063 g/mL with a high-density KBr solution and concentrated to ~1 mg protein/mL by ultrafiltration, before addition of EDTA or BC and ascorbate, which any Cu(II) was reduced to Cu(I), resulting in BC-Cu(I) complex formation. A further 0.6-ml sample of each diluted LDL solution was incubated with EDTA (1 ml added to a 100-µL volume of stock EDTA in water), for the reason explained in Results. Absorbances were measured at 480 nm, and the values obtained in the presence of EDTA were deducted from the values obtained in the presence of BC/ascorbic acid (the former values were typically 10% to 20% of the latter). Copper levels were determined by comparison with a CuSO₄ standard plot up to 100 µmol/L.

Electrophoresis of LDL

Native LDL, LDL carried through the equilibrium dialysis procedure in the presence of CuSO₄ and BHT, oxidized LDL, and chemically modified LDL samples (2 µg LDL protein) were allowed to diffuse into an agarose gel (Paragon Lipo gels, Beckman Instruments) for 5 minutes before the gels were run and stained according to the manufacturer’s instructions. Destaining was carried out in ethanol:water (45:55 [vol/vol]) for 1 minute or until background staining was no longer visible. After the gels were rinsed in water and dried, the migration of the LDL samples was measured and divided by the distance migrated by native LDL (REM).

Figure 1. Effect of buffers on copper binding to LDL. Copper binding to LDL (1 mg LDLP/mL) was measured at pH 7.4, in the presence of 10 µmol/L CuSO₄, 20 µmol/L BHT, and either unbuffered 150 mmol/L NaCl of pH 7.4 (approximately) or 150 mmol/L NaCl with 10 mmol/L phosphate, MES, MOPS, HEPES, or Tris, all at 4°C and pH 7.4. The number of copper ions bound per LDL particle (A) and the total concentrations of copper present in LDL-containing dialysis bags (B, stippled bars), in dialysis bags without LDL present (B, hatched bars), and in the bulk dialysis solution (B, open bars) are shown. The concentrations of copper present in LDL-containing dialysis bags have been adjusted for 1 mg LDL protein/mL. The concentration of CuSO₄ was not significantly different from the amount bound in MOPS buffer (Figure 1A). Use of HEPES buffer resulted in a 33% reduction in copper binding to LDL (P<0.001). Phosphate reduced copper binding by 70% (P<0.001) and Tris reduced binding by 78% (P<0.001). Copper binding to LDL in unbuffered NaCl solution (initially at pH 7.4) was slightly but
The addition of 1 mmol/L EDTA to samples without BC and ascorbate could lead to an underestimate of the bound absorbance at 480 nm, this light scattering in the absence of BC and ascorbate (LDL has an absorbance in the absence of BC and ascorbate). The attenuation increased greatly after 2 days as a result of light scattering, which occurred as a consequence of LDL aggregation. Although the increase was small over the time scale of the copper-binding assay, during which LDL is exposed to copper for 10 or 24 hours, it can still lead to significant error in the determination of bound copper. The aggregation became apparent to the naked eye where starting copper concentrations were high and were particularly evident due to precipitation.

**Measurement of Copper-Binding Sites on LDL in MOPS Buffer**

LDL (1 mg protein/mL) was subjected to equilibrium dialysis with 10 μmol/L CuSO₄ and 20 μmol/L BHT in MOPS buffer for up to 7 days. Figure 2 shows the attenuation (absorbance plus light scattering) at 480 nm that was due to LDL, in the absence of BC and ascorbate. The aggregation became apparent to the naked eye at late time points, as the LDL became turbid, but this turbidity was rapidly reversed (as was the light scattering) on the addition of BC and ascorbate (data not shown). Because the absorbance (A₄₈₀) values in the presence of BC and ascorbate have to be corrected by deducting the LDL absorbance in the absence of BC and ascorbate (LDL has an absorbance at 480 nm), this light scattering in the absence of BC and ascorbate could lead to an underestimate of the bound copper. The addition of 1 mmol/L EDTA to samples without BC and ascorbate could lead to an underestimate of the bound copper.

The optimum concentration of CuSO₄ for use in the copper-binding assay was 10 μmol/L, which saturated the copper-binding sites on LDL (Figure 3A). At initial levels of copper of ≥12.5 μmol/L, the copper present in the bulk solution decreased significantly in concentration before the peak copper binding to LDL was reached, with this effect being most pronounced when the highest starting concentrations of copper were used (data not shown). This loss of copper was due to precipitation and was apparent to the naked eye where starting copper concentrations between 20 and 100 μmol/L were tested.

Figure 3B shows the time taken for copper binding to LDL to reach equilibrium with use of the Spectra/Por 2.1 dialysis membrane and 10 μmol/L CuSO₄. In time-course experiments, a maximum binding of 38 to 42 copper ions per LDL particle was consistently reached by 10 hours of dialysis. The same level of maximum binding was obtained after 24 hours of dialysis across the Medicell membrane. The diffusion of copper across the membrane to replace that bound by LDL is presumably the rate-limiting step. The same degree of copper binding per LDL particle was observed at all tested concentrations.
trations of LDL (Figure 3C). The number of copper binding sites on LDL as the mean of 25 independent experiments (ie, the mean of 25 equilibrium dialysis copper-binding experiments that we have carried out in the absence of flavonoids or other inhibitors of copper binding) was 38.6 ± 0.7. To ensure that BHT did not influence copper binding to LDL (other than by preventing oxidation), experiments were performed that compared BHT with butylated hydroxyanisole (data not shown). In the absence of either antioxidant, mean copper binding to LDL was 50.9 ± 4.6 (this high figure was due to the considerable amount of oxidation that occurred; see below). The levels of copper binding to LDL observed in the presence of BHT and butylated hydroxyanisole were not significantly different (34.3 ± 2.1 and 37.2 ± 2.7 copper ions per LDL particle, respectively).

Copper Binding to LDL Isolated From Different Individuals

LDL was isolated from 12 different volunteers. Copper binding was measured for each sample in triplicate in a series of 3 experiments, each with LDL from 4 of the 12 donors. Individual values for copper binding per LDL particle were as follows (mean of triplicate): 40.7, 39.8, 39.1, and 37.0; 38.0, 33.6, 35.0, and 35.4; and 38.2, 37.4, 36.5, and 39.2. The mean of all samples was 37.5, with an SD of 2.1 and an SEM of 0.7. No significant differences were observed between means of data for each of the 3 groups of 4 samples each. The intra-assay coefficient of variation was 4%, and the interassay value was 5%. These results indicate that the differences observed between our copper-binding data and the data of others are not simply due to differences in the LDL samples used.

Effect of LDL Oxidation on Copper Binding

Although binding assays were carried out at 4°C in the presence of the antioxidant BHT, it was necessary to consider the potential effects of LDL oxidation on its copper-binding properties because of the requirement for relatively long-term exposure of LDL to copper ions. LDL that had not been oxidized, mildly oxidized LDL, moderately oxidized LDL (which contains the peak lipid hydroperoxide levels49), and highly oxidized LDL (as defined in Methods) differed in their copper-binding properties (Figure 4). After 10 hours of dialysis in Spectra/Por 2.1 tubing, moderately oxidized and highly oxidized LDL bound significantly more copper than did native LDL (P <0.001), but the binding of copper to LDL was not increased by mild oxidation. Moderately oxidized and highly oxidized LDLs were not saturated within the 10-hour time period (data not shown); therefore, the binding to these oxidized LDLs shown in Figure 4 is an underestimate of the true level of binding. Data for copper binding to oxidized LDL at saturation are not presented because at the long periods of time necessary, the concentration of free copper declined in the bulk solution, probably as a conse-
quence of precipitation. The extent of oxidation of the oxidized LDLs and LDL after dialysis in the presence of copper and BHT was assessed by agarose gel electrophoresis. REM values were as follows: 1.0 for native LDL and $1.4 \pm 0.2$, $1.9 \pm 0.1$, and $3.1 \pm 0.2$ for mildly, moderately, and highly oxidized LDL, respectively. The electrophoretic mobility of LDL dialyzed with the copper present consistently fell between that of native LDL and mildly oxidized LDL ($1.3 \pm 0.2$), implying that LDL is insufficiently oxidized during the equilibrium dialysis assay to alter its copper-binding properties compared with those of native LDL (see Figure 4). Lipid hydroperoxides as measured by a triiodide method were not increased during the assay (results not shown). Thiobarbituric acid–reactive substances were not suitable indicators of oxidation because they are dialyzable.

**Chemical Modifications of Histidyl and Lysyl Residues in ApoB-100**

It has been reported that copper binds in part to histidyl residues in apoB-100, on the basis of results involving their modification by diethylpyrocatecholate. Therefore, we have repeated these experiments, but using our method for measuring copper binding. Diethylpyrocatecholate was estimated spectrophotometrically to have modified $67 \pm 4\%$ of the histidyl residues in apoB-100 and significantly ($P<0.001$) reduced the number of copper ions bound per LDL particle from $37.9 \pm 1.6$ to $16.5 \pm 0.9$, a decrease of $56\%$.

To show that the association of copper ions with LDL, as measured by our method, was not simply a nonspecific effect of the net negative charge on LDL electrostatically attracting positively charged copper ions, we measured copper binding to acetylated LDL, which has a high net negative charge that is due to the modification of its lysyl residues. Acetylation did not increase the association of copper with LDL (which would be expected if the effect were due to the net charge of LDL) and actually reduced copper binding to $29.1 \pm 2.2$ per particle, a significant ($P<0.001$) decrease of $23\%$. The electrophoretic mobility relative to native LDL was $3.3 \pm 0.1$ for histidyl-modified LDL and $4.2 \pm 0.2$ for lysyl-modified LDL.

**Inhibition of Copper Binding by Antioxidants: Effect of Flavonoids**

To examine the inhibition by flavonoids of copper binding to LDL, flavonoids were added immediately from stock solutions in ethanol before the addition of copper to give a final flavonoid concentration of $10 \mu$mol/L and a final ethanol concentration of $0.25\%$ (vol/vol). As shown in Figure 5B, myricetin displayed the most potent inhibition of binding (82% inhibition, $P<0.001$), followed by quercetin (56% inhibition, $P<0.001$), and catechin (20% inhibition, $P<0.05$). Epicatechin, kaempferol, and morin at this concentration did not significantly affect copper binding to LDL. Chrysin was also tested but was found to cause a significant loss of free copper from solution by precipitation. Histidine, a compound with a high affinity for copper ions, was included at a concentration of $20 \mu$mol/L to act as a positive control and was found to reduce copper binding to LDL by $>90\%$.

We examined whether the inhibition by flavonoids of copper binding to LDL was due to irreversible modification of copper-binding sites on LDL. LDL samples were predialyzed for 12 hours in Spectra/Por 2.1 tubing in the absence of copper, with or without quercetin or myricetin (10 $\mu$mol/L). A second dialysis was then performed in the presence of copper as usual (Figure 5C). For samples that had been preincubated with flavonoids, this second dialysis was carried out in the absence of flavonoids. For samples that had been preincubated in the absence of flavonoids, quercetin or myricetin ($10 \mu$mol/L) was included in the second dialysis. Control LDL did not encounter flavonoids at either dialysis stage. LDL that had been preincubated with either quercetin or myricetin bound significantly more, rather than less, copper than LDL that had been preincubated in the absence of flavonoids ($P<0.05$), when quercetin or myricetin was absent from the second dialysis. This suggests that copper can form a tertiary complex with LDL-bound flavonoids but can be easily removed from LDL or LDL-associated flavonoids by free flavonoids in solution.

**Discussion**

Our results demonstrate the presence of $38.6 \pm 0.7$ (n=25 independent experiments) copper-binding sites per LDL particle. MOPS/NaCl was the most suitable buffer for the measurement of copper binding to LDL by equilibrium dialysis. Tris, phosphate, and, to a lesser extent, HEPES presumably formed complexes with copper ions and thereby reduced copper binding to LDL. HEPES and Tris have been reported to have a low affinity for copper, as determined by displacement of a pH titration curve when copper was present at a concentration equivalent to that of the buffer. In systems used to measure copper binding to LDL, however, the buffer is present in great excess over the copper (1000-fold excess in our system). Therefore, a buffer normally considered to have a low affinity for copper can still interfere significantly with copper-binding assays. We cannot exclude the possibility that some of the buffers that inhibit copper binding to LDL may do so by blocking copper-binding sites on the lipoprotein.

The number of binding sites increased with LDL oxidation, although the low level of oxidation occurring during the equilibrium dialysis procedure with copper in the presence of BHT was not sufficient to affect the copper binding properties of LDL (Figure 4). Increased binding of divalent cations, such as manganese(II), by LDL on oxidation or malondialdehyde modification has been reported by others and has been suggested to be due to the modification of lysyl residues, which may prevent their interaction with aspartyl and glutamyl residues in apoB-100 and thus increase the binding of divalent cations to these acidic residues. However, this does not explain the increased copper-binding capacity of oxidized LDL, inasmuch as the acetylation of lysyl residues significantly decreased copper binding to LDL. The decrease in copper binding associated with acetylation (and the concomitant increase in net negative charge) of LDL also suggests that the increased copper-binding capacity of oxidized LDL is not purely charge dependent. It is possible that increased copper binding by oxidized LDL is a consequence of the proteolysis of apoB-100 that occurs during oxidation, which may facilitate conformational changes that result in the exposure of new sites with copper-binding potential. It is also possible that copper may bind to the carboxylate groups of fatty acid derivatives generated by lipoprotein-associated...
phospholipase A<sub>2</sub> during LDL oxidation<sup>61,62</sup> or to oxidatively modified aminoacyl residues in apoB-100.<sup>63</sup>

We have demonstrated a 56% reduction in copper binding after the modification of histidyl residues in apoB-100 and have thereby confirmed the findings of Chen and Frei<sup>22</sup> and Wagner and Heinecke.<sup>23</sup> Acetylation of lysyl residues resulted in a 23% reduction in copper binding. This might conceivably be due to a conformational change in apoB-100. Copper ion binding to the enol ether double bond of plasminogen may account for some of the remaining copper-binding sites on LDL.<sup>64</sup>

As far as we are aware, we report the first reversible copper-dependent aggregation of LDL. LDL became aggregated during prolonged incubation with copper in the presence of BHT. BC and EDTA were both capable of completely reversing the aggregation. Although the mechanism of this aggregation is unclear, it may be related to the reversible copper-dependent aggregation of amyloid-β peptides.<sup>65</sup>

The number of copper binding sites previously reported on LDL varies enormously and appears to depend primarily on the choice of buffer and the method of separating bound from free copper (Table). Separation by ultrafiltration<sup>22,23,27,28</sup> has led to estimates quite different from ours and may be confounded by interactions of copper ions with LDL gel layers. Gel-layer formation results in a barrier to filtration above the membrane, greatly decreasing the speed of ultrafiltration and making the passage of small molecules into the filtrate increasingly difficult.<sup>66,67</sup>

Column chromatography gives very low estimates of copper binding to LDL.<sup>24-26,30</sup> There is likely to be a problem with this technique, inasmuch as copper ions bind to Sephadex during column chromatography. This can be readily seen by passing LDL-copper complexes through Sephadex G-25 PD-10 columns (Pharmacia Biotech) and eluting with BC and ascorbate after the LDL has emerged and the column has been eluted with several times its bed volume. Copper-BC complex formation is visible only at the head of the column, even if the BC/ascorbate solution is passed through the column in reverse, ie, from the foot upward.

Precipitation of LDL with the use of methanol and HDL cholesterol precipitant has also been used to separate free from lipoprotein-bound copper<sup>31</sup> and gives an estimate of ≈100 copper ions bound per LDL particle. This technique may have measured copper binding to denatured LDL, and it has been speculated that copper may have been adsorbed to the precipitate.<sup>32</sup>

Kinetic analysis based on the oxidation of LDL by copper has been used to examine copper binding to LDL,<sup>32,68,69</sup> but this would measure only redox-active binding sites.

Using dialysis to separate free from lipoprotein-bound copper in the absence of a bulk pool of copper in the dialysis buffer<sup>26,29</sup> may result in the dissociation of some of the copper from the LDL.

Equilibrium dialysis has been used by other workers,<sup>23</sup> but the procedure was carried out in a HEPES buffer, which we find decreases the binding of copper to LDL (Figure 1). Also, the dialysis was performed for only 4 hours, which may have been insufficient to saturate the copper binding sites on LDL.

For the reasons given above, we believe that the equilibrium dialysis technique described in the present study has advantages over other methods that measure copper binding to LDL.

Quercetin and myricetin substantially inhibited copper binding to LDL, with myricetin being significantly more effective than quercetin (<i>P</i> < 0.001). These 2 flavonols possess a carbonyl group at the 4 position and hydroxyl groups at the 3 and 5 positions. Either of these hydroxyl groups could act in concert with the carbonyl oxygen to chelate transition metal ions.<sup>70</sup> However, kaempferol and morin were unable to inhibit copper binding to LDL despite the presence of a carbonyl group at C-4 and hydroxyl groups at C-3 and C-5, suggesting that these features alone are insufficient to allow significant copper binding to a flavonoid. Adjacent hydroxyl groups on the B ring have also been implicated in transition metal binding by flavonoids,<sup>47,71,72</sup> but these are present in catechin and epicatechin (both of which lack a C-4 carbonyl and a C-2 to C-3 double bond) without enabling these flavan-3-ols to bind copper to any great extent at the concentration used in the present study. It is likely that the C-ring flavone structure acts in concert with B-ring vicinal hydroxyl groups to facilitate copper complex formation by the effective flavonoids, possibly after hydrogen abstraction from a B-ring hydroxyl group.<sup>70</sup> The superior ability of myricetin over quercetin to decrease copper binding to LDL may be due to the presence of the additional hydroxyl group on the B ring, providing additional stabilization of a 4<sup>+</sup> anion through hydrogen bond formation and through resonance stabilization. Also, the presence of 3 sequential hydroxyl groups in the B ring increases the probability of copper chelation by 2 adjacent oxygen atoms.

The antioxidant activity of flavonoids depends on their free radical–scavenging activity as well as their ability to chelate copper or iron, inasmuch as kaempferol and morin inhibit lipoprotein oxidation<sup>34,35,47</sup> even though they do not inhibit copper binding to LDL. Once attacked by free radicals, oxidized flavonols might continue to act as antioxidants by chelating pro-oxidant metal ions and holding them in an inactive form.

The occurrence of transition metal ion binding by flavonoids in vivo remains to be determined, but sequestration of pro-oxidant metal ions may play a part in the possible protection against atherosclerosis by dietary flavonoids. Because flavonoids can be extensively metabolized in vivo,<sup>73,74</sup> the ability of flavonoid metabolites to bind transition metals warrants examination. It has been suggested that by analogy with other inflammatory sites, atherosclerotic lesions may have an acidic extracellular pH, and it is known that some metal-carrying proteins release transition metal ions under acidic conditions.<sup>17</sup> Because pH affects metal binding to flavonoids,<sup>44,71,74</sup> studies could be focused on flavonoids having structures that are likely to confer the highest metal-binding activity at mildly acidic pH.

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