# $Cu^{2+}$-mediated oxidation of dialyzed plasma: effects on low and high density lipoproteins and cholesteryl ester transfer protein

**Zbigniew Zawadzki, Ross W. Milne, and Yves L. Marcel**[1]

Laboratory of Lipoprotein Metabolism, Clinical Research Institute, Montreal, Quebec, H2W 1R7 Canada

**Abstract** We previously reported that the expression of an epitope of apolipoprotein B (apoB), mapped to the C-terminus and defined by antibody $B_{sol}7$, increased during $Cu^{2+}$-mediated oxidation of isolated low density lipoprotein (LDL). We describe now the properties of $B_{sol}7$ as a marker of LDL oxidation in whole plasma in relation to other effects of oxidative treatment of plasma, such as the distribution of apoA-I and cholesteryl ester transfer protein (CETP). In dialyzed plasma, no LDL oxidation was detected at $Cu^{2+}$ concentrations (5 $\mu$M) sufficient for extensive oxidation of isolated LDL. At a higher $Cu^{2+}$ concentration (50 $\mu$M) , an increased expression of the $B_{sol}7$ epitope was observed; at 250 $\mu$M $Cu^{2+}$, other evidence of LDL oxidation was found. The pattern of LDL response to $Cu^{2+}$ observed in dialyzed plasma could be reproduced by adding 3% bovine serum albumin to isolated LDL. We demonstrate that the effect of albumin most likely results from its ability to bind copper ions. Incubation of plasma with increasing concentrations of $Cu^{2+}$ resulted first in the disappearance of $\alpha_2$-migrating HDL, the usual carrier of CETP; free CETP and high molecular weight apoA-I-containing particles were also generated during oxidation. Addition of oxidized, but not native, LDL to plasma resulted in a transfer to LDL of some of the CETP initially associated with apoA-I. In conclusion, the increased immunoreactivity of the $B_{sol}7$ epitope was the most sensitive parameter of LDL oxidation, but other parameters, such as the presence of $\alpha_2$-HDL and CETP-lipoprotein associations were even more sensitive evidence of lipoprotein oxidation. —**Zawadzki, Z., R. W. Milne, and Y. L. Marcel.** $Cu^{2+}$-mediated oxidation of dialyzed plasma: effects on low and high density lipoproteins and cholesteryl ester transfer protein. *J. Lipid Res.* 1991. **32:** 243–250.

**Supplementary key words** monoclonal antibodies • LDL oxidation • apoprotein B immunoreactivity • plasma CETP distribution • $\alpha_2$-migrating HDL

The role of oxidative modification of low density lipoprotein in atherogenesis has been substantiated by several studies (1–3), albeit not unequivocally (4). The efforts by many laboratories to characterize LDL oxidation and assess its physiological role have generated a considerable amount of information on the mechanism of the process (5–13) and fairly detailed descriptions of its products (7, 8, 10, 14, 15). Much of that information has been accumulated using a simple model of LDL oxidation, consisting of the isolated lipoprotein incubated with either oxygen-containing buffers (5, 16), copper ions (8, 12), or endothelial cells (6, 14, 17). The situation in vivo is, of course, much more complex and the obvious question arises whether the observations made upon oxidation of isolated LDL apply to a more physiological environment, such as human plasma.

We have recently reported (18) the changes in apolipoprotein B immunoreactivity during oxidation of isolated LDL. Using several anti-apoB monoclonal antibodies we found that the expression of three epitopes mapped to the N-terminal region, middle part, or LDL-receptor binding domain of apoB decreased gradually during the oxidation, while the expression of another epitope ($B_{sol}7$) located at the C-terminus of the apoB molecule increased markedly during the first stages of oxidative processes. We suggested that the $B_{sol}7$ epitope could serve as an immunochemical marker of LDL oxidation.

It has been reported from our laboratory (19) that the oxidative treatment of high density lipoprotein results in changes in apolipoprotein A-I immunoreactivity and also in apoA-I cross-linking. An $\alpha_2$-migrating subfraction of HDL [also referred to by others as pre-$\beta$-HDL (20)] has been shown to be particularly responsive to oxidative conditions. This $\alpha_2$-HDL was also found to contain most of plasma CETP in complexes of about 150 kDa (21). Since the decreased positive charge of lipoproteins facilitates

Abbreviations: BHT, butylated hydroxytoluene; NCP, nitrocellulose paper; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIA, radioimmunoassay; LpA-I, apolipoprotein A-I-containing lipoproteins; $\alpha_2$-HDL, high density lipoprotein with $\alpha_2$ agarose electrophoretic mobility; CETP, cholesteryl ester transfer protein; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

[1]To whom reprint requests should be addressed at: Clinical Research Institute, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7 Canada.

their binding to CETP as demonstrated with the acetylation of LDL apoB (22), and since oxidation generates LDL with reduced numbers of positively charged lysine residues in apoB (8), we speculated that the formation of oxidized LDL may affect the normal binding of CETP to $\alpha_2$-HDL; if true, this could be expected to have possible physiological consequences. In the present paper we report both our attempts to use the $B_{sol}7$ marker in experiments carried out in the whole plasma and our observations on the effects of oxidative treatment of plasma on CETP and its physiological carrier, the $\alpha_2$-migrating fraction of HDL.

## MATERIALS AND METHODS

### Plasma and lipoprotein samples

Blood from normolipidemic humans was collected into EDTA-containing Vacutainers and centrifuged for 15 min at 2000 *g* to separate plasma which was used in subsequent experiments within 3 days from blood drawing. ApoB concentration was measured by RIA using antibody 4G3 (see below). Plasma aliquots were used to isolate low density lipoprotein by sequential ultracentrifugation (1.020 g/ml < d < 1.050 g/ml). Isolated LDL was dialyzed against PBS, 10 $\mu$M EDTA, and protein was measured according to Lowry et al. (23).

### Oxidative treatment

For the oxidation of isolated LDL, the procedure of Steinbrecher (8) was used, as described previously (18). The same protocol was used in experiments in which bovine serum albumin was added to the incubation mixture or when whole plasma was exposed to $Cu^{2+}$ ions. Prior to treatment, samples were dialyzed against EDTA-free PBS. The concentration of copper ions varied from 5 $\mu$M to 500 $\mu$M. In control incubations, which did not contain $Cu^{2+}$, 1 mM EDTA and 40 $\mu$M BHT were present. The treatment was arrested by addition of 1 mM EDTA and 40 $\mu$M BHT, followed by dialysis against PBS with the same additives.

### Monoclonal antibodies and antisera[2]

The production and characterization of monoclonal antibodies 4G3 and $B_{sol}7$ against apoB (24–27), 4H1 against apoA-I (28), and TP-2 against CETP (29) have been described earlier. TP-2 was labeled with $^{125}I$ using the chloramine T method (30). Affinity-purified rabbit anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD) and iodinated using the chloramine T method (30).

[2]Samples of antibodies 4G3, $B_{sol}7$, 4H1, and TP-2 can be obtained from our laboratory.

### Solid phase radioimmunoassay of apoB

The assay has been described earlier (18, 27). Polystyrene wells (Removawells, Dynatech Laboratories Inc., Alexandria, VA) were used for immobilization of normal LDL, which competed for the monoclonal antibody with different concentrations of the sample to be tested. The amount of monoclonal antibody bound to the solid phase was determined using $^{125}I$-labeled rabbit anti-mouse IgG. Maximum binding was determined in wells to which no competing soluble antigen was added. The results are expressed as $B/B_{max}$ ratio. Each point is the mean of two measurements (CV < 10%).

### Polyacrylamide gel electrophoresis

The system of Laemmli (31) was used for 1.5-mm-thick 5–15% polyacrylamide gradient gels containing SDS. The sample buffer contained 10 $\mu$M Tris (pH 6.8), 3% SDS, 20% glycerol, 10% 2-mercaptoethanol, and bromophenol blue marker. The electrophoresis was carried out at 20 mA per gel until the marker reached the bottom of the gel. The proteins were either stained with Coomassie blue R-250 or transferred to nitrocellulose paper (0.45 $\mu$m pore size, Millipore) according to Towbin, Staehelin, and Gordon (32). The transfer buffer was 62.5 mM boric acid (pH 8.0) 30% methanol. Nondenaturing 2–16% and 4–30% polyacrylamide gradient gels (Pharmacia, PAA 2/16 and PAA 4/30, Sweden) were used according to Gambert et al. (33). The electrophoresis and transfer to NCP have been described earlier. The immunodetection of proteins in NCP was carried out as described earlier for anti-apoB (24), anti-A-I (28), and anti-CETP (21) monoclonal antibodies.

### Agarose gel electrophoresis

Paragon Lipo gels (Beckman) were used; electrophoresis, fixing, and lipid staining were performed according to the instructions of the manufacturer. The transfer to NCP and immunodetection have been described earlier (18).

### Albumin studies

Two commercial preparations of bovine serum albumin (Fraction V, from bovine serum and Fraction V, fatty acid-free, from bovine serum, both from Boehringer, Mannheim, Germany) were used, as well as a charcoal-treated albumin; all gave the same results in incubations with LDL and $Cu^{2+}$. For saturation with fatty acids the commercial fatty acid-free preparation was additionally treated with charcoal to remove the residual fatty acids, using the method of Chen (34). The saturation of albumin with fatty acids was performed using the Celite method of Spector and Hoak (35); the Celite was removed from albumin solution by filtration.

### Fast protein liquid chromatography

Plasma (0.5 ml) before or after incubation with $Cu^{2+}$ was injected onto a Superose 12 column connected to the Pharmacia FPLC system. The elution with 10 mM Tris, 150 mM NaCl, 0.01% EDTA, 1 mM $NaN_3$, pH 8.0, was carried out at the rate of 30 ml/h. Fractions of 0.5 ml were collected and 3-$\mu$l aliquots of each fraction were applied on nitrocellulose paper. The NCP was processed as described earlier for anti-CETP antibody TP-2 (21).

## RESULTS AND DISCUSSION

### LDL during plasma oxidation

When the standard oxidation procedure of incubating the LDL sample in EDTA-free PBS (200 $\mu$g LDL protein/ml) with 5 $\mu$M $Cu^{2+}$ for 18 h was applied to fresh, dialyzed human plasma, LDL and VLDL were not affected. That could be judged by the lack of effect on LDL agarose mobility (not illustrated), the absence of apoB degradation as demonstrated by SDS gels (**Fig. 1**), and the unchanged immunoreactivity with antibodies $B_{sol}7$ and 4G3 (**Fig. 2A and B**). This was in sharp contrast with the effect of similar incubations on isolated LDL, as described earlier (18). Extending the incubation time up to 48 h did not affect the results. When the copper ion concentration in the incubation mixture was raised to 50 $\mu$M, we were still unable to detect any change in LDL agarose mobility, apoB SDS-PAGE migration, or LDL immunoreactivity with 4G3. But, interestingly, at this concentration of $Cu^{2+}$, the RIA using antibody $B_{sol}7$ detected a substantial increase in immunoreactivity of LDL (Fig. 2A). Further increases of $Cu^{2+}$ concentration (250–500 $\mu$M) did not bring about any additional change in the

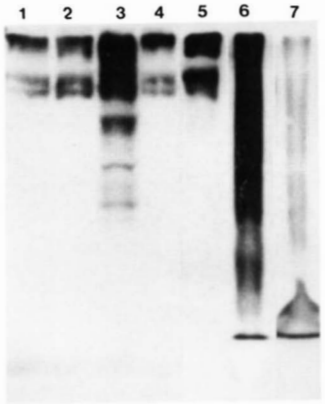


**Fig. 1.** Immunoblot of an SDS-polyacrylamide gradient gel (5–15%) obtained with anti-apoB antibody 4G3. Plasma was incubated for 24 h with: 5 $\mu$M $Cu^{2+}$ (lane 1), 50 $\mu$M $Cu^{2+}$ (lane 2), 500 $\mu$M $Cu^{2+}$ (lane 3), and with no $Cu^{2+}$ (lane 4); isolated LDL (lane 5) was incubated with 5 $\mu$M $Cu^{2+}$ for 3 h (lane 6) and 18 h (lane 7).

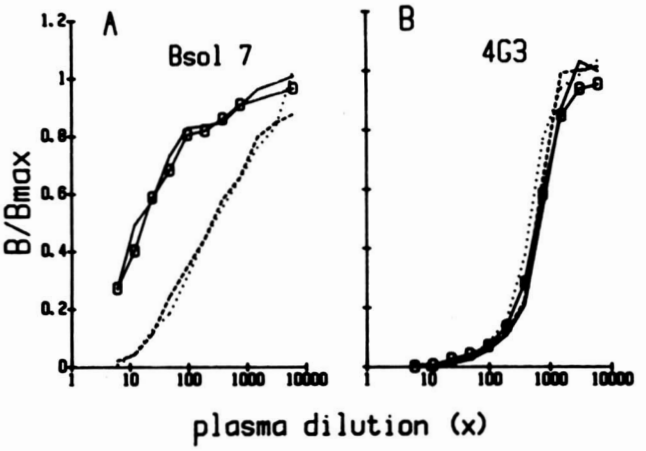


**Fig. 2.** Competitive RIA with (A) antibody $B_{sol}{}^7$ and (B) antibody 4G3. The competing antigen was plasma incubated for 18 h with: (—) EDTA + BHT; (-□-) 5 $\mu$M $Cu^{2+}$; (---) 50 $\mu$M $Cu^{2+}$; or (····) 500 $\mu$M $Cu^{2+}$.

$B_{sol}7$ RIA displacement curve, but an increase in LDL mobility in agarose could be detected both by lipid staining and immunodetection with 4G3 (not illustrated). The Western blotting of SDS-polyacrylamide gel with both 4G3 and $B_{sol}7$ detected fragmentation of apoB in these samples, although the extent of changes was far from that observed after $Cu^{2+}$ treatment of isolated LDL for the same time (Fig. 1).

Since we have previously observed (18) that incubation of isolated VLDL with $Cu^{2+}$ does not affect its immunoreactivity with either 4G3 or $B_{sol}7$, we assume that the effects of 50 $\mu$M and 250 $\mu$M $Cu^{2+}$ described above result from LDL, rather than VLDL, modification.

In order to explain the mechanism preventing the LDL in plasma from being oxidized during incubation with copper ions, we repeated the oxidative treatment of isolated LDL in the presence of 3% bovine serum albumin. The results were virtually identical with those reported above for plasma. Again, at $Cu^{2+}$ concentration of 5 $\mu$M no changes could be detected in LDL agarose mobility (**Fig. 3**) or any other of the parameters studied; at 50 $\mu$M $Cu^{2+}$ an increase in immunoreactivity with $B_{sol}7$ was the only effect observed, and at 250–500 $\mu$M $Cu^{2+}$ it was accompanied by increased agarose mobility of LDL and some limited fragmentation of apoB (not illustrated).

### HDL and CETP during plasma oxidation

The next step was to verify whether the gradual $Cu^{2+}$-concentration-dependent effect that the treatment has on LDL could also be seen in HDL. **Fig. 4A** shows changes in apoA-I distribution during incubation of plasma with $Cu^{2+}$. It can be seen that at 50 and 500 $\mu$M $Cu^{2+}$ there is a decrease in intensity of the apoA-I signal detected in the molecular weight range of 100,000 to 150,000; at the same

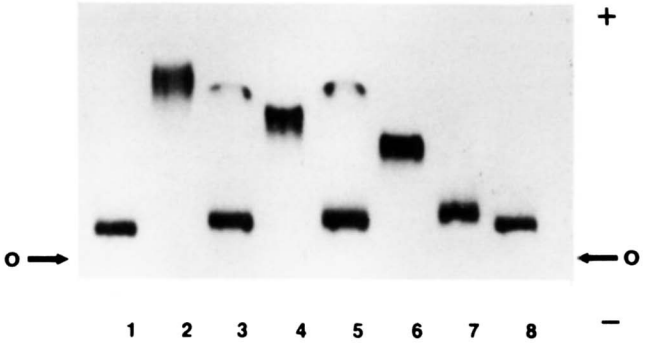


**Fig. 3.** Lipid staining of an agarose gel after electrophoresis of LDL incubated with 5 $\mu$M $Cu^{2+}$. LDL incubated for 24 h with EDTA + BHT (lane 1), $Cu^{2+}$ (lane 2) and $Cu^{2+}$ in the presence of 3% BSA (lane 3); 6 h incubation with $Cu^{2+}$ (lane 4) or $Cu^{2+}$ + 3% BSA (lane 5); 3 h incubation with $Cu^{2+}$ (lane 6); 1 h incubation with $Cu^{2+}$ (lane 7); time = 0 incubation with $Cu^{2+}$ (lane 8). The additional electronegative band in lanes 3 and 5 represents the BSA; the intensity of its Sudan black staining depends on the content of fatty acids and may change as the oxidation proceeds.

time more apoA-I-containing material can be found in the higher molecular weight range, as compared with the plasma before incubation.[3]

We reported recently (21) that the apoA-I-containing lipoproteins of molecular weight ranging from 129,000 to 154,000 are the CETP-carrying particles which in agarose migrate as an $\alpha_2$-HDL fraction (in contrast with the $\alpha_1$-migrating bulk of HDL) and in Fig. 4B we show that CETP in fresh plasma is indeed detected in that particular molecular weight range (lane 1). During incubation with increasing concentrations of copper ions, the CETP signal is transferred to a band of lower molecular weight (lanes 2 and 3), co-migrating with partially purified CETP. The identity of that band was confirmed by gel filtration. Samples of dialyzed plasma before and after treatment with 500 $\mu$M $Cu^{2+}$ were applied on a Superose 12 column and eluted in an FPLC system. The CETP-containing fractions of the eluate were identified by immunodetection on a nitrocellulose dot blot. The $Cu^{2+}$ treatment shifted the CETP signal toward the lower molecular weight region of the eluate (not illustrated), coinciding with the partially purified CETP sample eluted from the same column. Interestingly, we previously reported (21) that upon aging of plasma a decrease in the molecular weight of CETP bands occurs, identical to that seen in Fig. 4B; these effects are observed also in the presence of protease inhibitors, confirming that an oxidative mechanism may be an underlying cause. Thus, it appears that under oxidative conditions the CETP-carrying particles are destroyed; the bonds between CETP and the lipoprotein are broken, resulting in the release of CETP.

Immunoblots of agarose gels provide further support for that mechanism. CETP in plasma is present mostly, or even solely, in the $\alpha_2$-migrating subfraction of LpA-I. **Fig. 5** shows that the intensity of the apoA-I signal in the $\alpha_2$-HDL band is reduced even in the presence of 5 $\mu$M $Cu^{2+}$ and the band completely disappears at 50 or 500 $\mu$M $Cu^{2+}$.

At this point we can only speculate about the reasons for that particular responsiveness of $\alpha_2$-migrating HDL to oxidative conditions. The lipids present in that HDL subfraction may contain fatty acids particularly sensitive to oxidation. On the other hand, one of the striking features of $\alpha_2$-HDL is its exceptionally low content of lipids (20). These particles, consisting in over 90% of proteins are probably discoidal and have very little, if any, of the lipid core. The antioxidant properties of several lipophilic substances present in the lipid core of lipoprotein particles (e.g., $\beta$-carotene or vitamin E in normal humans, probucol in treated subjects) are well established. Due to its composition, the $\alpha_2$-HDL subfraction may be depleted of that antioxidant protection.

### Plasma dialysis and oxidation

Plasma used in the above experiments had been dialyzed prior to the $Cu^{2+}$ treatment in order to remove the EDTA. The concomitant removal of other low molecular weight components by dialysis may have altered the plasma sensitivity to oxidation. For instance, diet-derived ascorbate is known (37) to provide an important physiological protection against lipid peroxidation. To take account of the possible effects of dialysis on plasma susceptibility to oxidation, we repeated the $Cu^{2+}$ treatment using nondialyzed serum instead of dialyzed plasma and we analyzed the products on nondenaturing polyacrylamide gradient gels. The resulting changes in apoA-I and

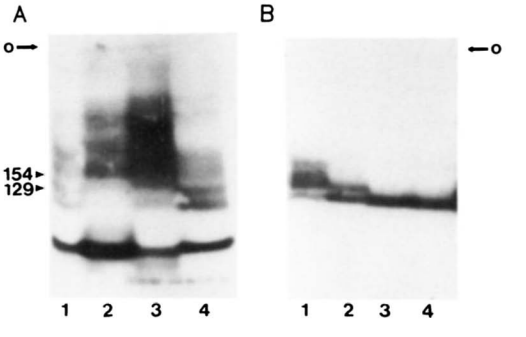


**Fig. 4.** Immunoblots of nondenaturing polyacrylamide gradient gels (4–30%) obtained with (A) anti-apoA-I antibody 4H1 or (B) anti-CETP antibody TP-2. A: plasma incubated for 6 h with 5 $\mu$M (lane 1), 50 $\mu$M (lane 2), 500 $\mu$M (lane 3) $Cu^{2+}$, or with EDTA + BHT (lane 4). B: Plasma (lane 1) incubated for 6 h with 50 $\mu$M (lane 2) or 500 $\mu$M (lane 3) $Cu^{2+}$. Partially purified CETP in lane 4.

[3]The band seen at 56 kDa has been described earlier (36). It may represent the apoA-I dimer or some other form of free apoA-I, present in plasma only in minor quantities; the intensity of the band reflects high immunoreactivity of that apoA-I form with 4H1.

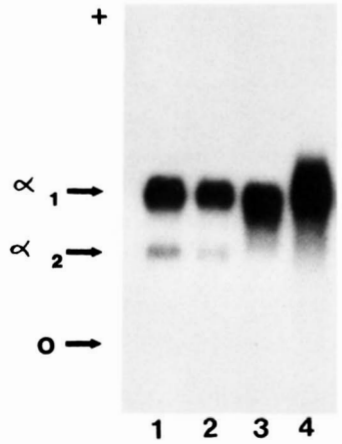


**Fig. 5.** Immunoblot of an agarose electrophoregram obtained with anti-apoA-I antibody 4H1. Plasma (lane 1) was incubated for 6 h with 5 $\mu$M (lane 2), 50 $\mu$M (lane 3), or 500 $\mu$M (lane 4) $Cu^{2+}$.

CETP distribution were identical to those presented in Fig. 4A and 4B (not illustrated) with one important quantitative difference: these changes appeared only at $Cu^{2+}$ concentrations higher than 100 $\mu$M. This shows indeed that, the dialyzable plasma components provide an additional protection against the $Cu^{2+}$-mediated oxidation; that protection could be overcome by using higher concentrations of copper ions. On the other hand, in our experiments the dialyzable components accounted only partially for the protection against $Cu^{2+}$-mediated modification since the oxidation of LDL in dialyzed plasma still required higher concentrations of $Cu^{2+}$ than in the case of isolated lipoproteins.

### CETP association with oxidized LDL

In order to test the hypothesis that oxidation of LDL might also influence the distribution of CETP in plasma because of the increased negative charge of oxidized LDL, we incubated an aliquot of plasma with exogenous oxidized LDL obtained by $Cu^{2+}$ treatment of isolated lipoprotein. **Fig. 6** shows the results of immunodetection of CETP after nondenaturing gradient gel electrophoresis of plasma incubated for 18 h with either native or oxidized LDL at the same protein concentration (40 $\mu$g/ml). The appearance of a CETP signal corresponding to the LDL band could be seen in the sample containing oxidized, but not native, LDL consistent with the role of a lipoprotein ionic charge in CETP binding. One would expect that similar CETP binding by LDL will occur in plasma incubated with $Cu^{2+}$ concentrations high enough to generate oxidized LDL. The experiments testing that possibility gave, however, poorly reproducible results. It is possible that different levels of antioxidant substances in various plasma samples used may have contributed to that variability. The difference, seen in Fig. 6, between the CETP migration in nonincubated (lane 5) and incubated plasma (lanes 2–4) is another illustration of the shift in the CETP signal towards lower molecular weight particles observed earlier (21) during plasma aging or incubation at 37°C, and, in the present study, during incubation with $Cu^{2+}$ (Fig. 4B).

### Albumin and oxidation: pre-saturation with fatty acids

In our experiments the attenuated response of LDL in dialyzed plasma to the presence of $Cu^{2+}$ could be mimicked by adding 3% albumin to isolated LDL, suggesting that it is mainly the albumin that is responsible for the nondialyzable part of LDL protection against the oxidative treatment in plasma. This protective mechanism may reflect the capability of albumin to complex $Cu^{2+}$ ions (38). On the other hand, albumin with its affinity for fatty acids might interfere with the initiating step of oxidation if the liberation of a fatty acid by phospholipase $A_2$ was indeed a prerequisite for LDL oxidation, as postulated by Parthasarathy et al. (6). However, the study by Steinbrecher and Pritchard (39) casts doubt on Parthasarathy's hypothesis and suggests that the phosphatidylcholine hydrolysis is a result of oxidation and not its initiating step. To obtain more information on the possible mechanism of albumin inhibition of $Cu^{2+}$-mediated oxidation, we performed several experiments in which we varied the content of different fatty acids and metal ions in albumin samples prior to their use in incubations with LDL and $Cu^{2+}$.

To deliver fatty acids to albumin, we incubated aliquots of the fatty acid-free BSA solution with varying amounts of fatty acid-coated Celite (35). In these experiments we used five different fatty acids: two saturated (palmitic and

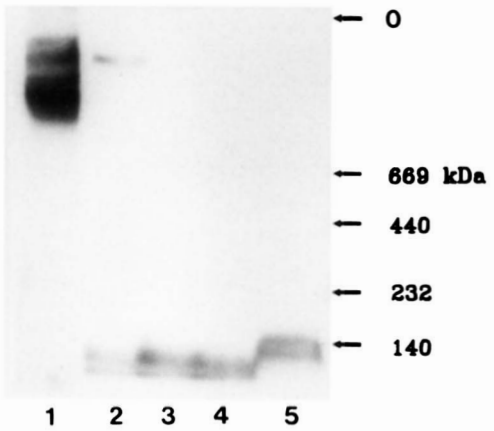


**Fig. 6.** Immunoblot of a nondenaturing polyacrylamide gradient gel (2–16%) obtained with anti-apoB antibody 4G3 (lane 1) or anti-CETP antibody TP-2 (lanes 2–5). Plasma (lane 5) was incubated for 18 h at 37°C with oxidized LDL (lanes 1 and 2), native LDL (lane 3), or without any additives (lane 4).

stearic) and three unsaturated (arachidonic, elaidic, and linolenic). The incubations were performed at two different molar ratios of fatty acid to albumin 5:1 and 20:1. The fatty acid-enriched albumin was then used in incubations with LDL and $Cu^{2+}$. None of the fatty acids incubated with BSA changed dramatically the inhibitory role of albumin in LDL oxidation. The most significant effect was observed with linolenic acid. As seen in **Fig. 7A**, linolenate-enriched BSA was less efficient in protecting LDL against 100 $\mu$M $Cu^{2+}$ than fatty acid-free albumin. When BSA containing higher concentrations of linolenic acid was incubated with LDL, even in the absence of $Cu^{2+}$ there was an increase in LDL agarose electrophoretic migration (not accompanied by evidence of apoB fragmentation in SDS gels), due probably to the delivery of fatty acid molecules to LDL; the same effect could also be seen with high concentrations of other fatty acids studied. The enrichment of BSA with either of the saturated fatty acids did not result in any detectable change in LDL protection against oxidation.

### Albumin and oxidation: pre-saturation with metal ions

We reasoned that if the antioxidant properties of BSA simply reflected the ability of albumin to bind $Cu^{2+}$ ions, preincubation with copper or other divalent ions should diminish the protective capability of BSA. We therefore preincubated aliquots of 3% BSA solution with varying concentrations of copper, zinc, or calcium ions. After dialysis to remove the unbound ions, the albumin was used in standard incubations with LDL and $Cu^{2+}$. Preincubation of albumin with 5 or 50 $\mu$M $Cu^{2+}$ did not affect its behavior during subsequent incubation with LDL. On the other hand (Fig. 7B), the albumin preincubated with 150 $\mu$M $Cu^{2+}$ brought about a substantial oxidation of LDL even in the absence of additional copper in the incubation mixture. All albumin samples pre-treated with higher concentrations of $Cu^{2+}$ readily oxidized LDL without any further addition of copper (not illustrated). Since the preincubated samples were extensively dialyzed before addition of LDL, these results confirm that BSA is capable of binding $Cu^{2+}$ ions; they also demonstrate that the bound ions are capable of oxidizing LDL at concentrations ($>$ 100 $\mu$M) similar to those required in experiments with BSA that had not been pre-treated. The effect of the $Cu^{2+}$ ions added during preincubation of the BSA and during incubation with LDL was additive, i.e., adding the given amount of copper at either of these two stages or dividing it between them brought about the same increase in LDL migration and apoB fragmentation. Preincubation of albumin with calcium ions (500 $\mu$M) did not affect the minimal concentration of $Cu^{2+}$ required to cause an LDL oxidation detectable by agarose electrophoresis. On the other hand, pretreatment with zinc ions rendered albumin slightly less capable of protecting LDL against $Cu^{2+}$-mediated oxidation (Fig. 7B), but $Zn^{2+}$ concentrations as high as 10 mM had only a moderate effect (higher concentrations could not be studied because of precipitation of BSA).

Since the pre-treated albumin solution was dialyzed before its subsequent use, we also dialyzed a control, non-treated aliquot of commercial fatty acid-free BSA. It turned out (Fig. 7C) that the dialysis itself can modify the albumin properties in incubations with LDL and $Cu^{2+}$, weakening its antioxidant protection. The changes shown in Fig. 7C may reflect a removal of a substance capable of interfering with the $Cu^{2+}$-mediated process (e.g., citrate

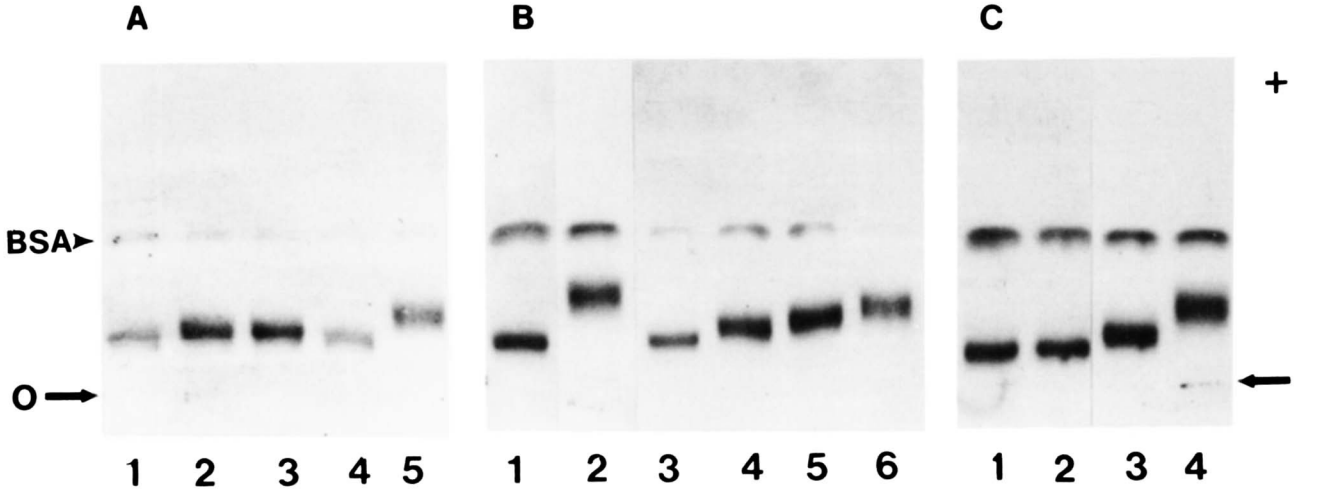


**Fig. 7.** Lipid staining of an agarose electrophoregram. A: LDL incubated with 100 $\mu$M $Cu^{2+}$ in the presence of 3% regular BSA (lane 1) or BSA pre-incubated with palmitic acid (lane 2), stearic acid (lane 3), elaidic acid (lane 4), and linolenic acid (lane 5) (fatty acid/BSA molar ratio 5:1). B: LDL incubated with 3% regular BSA (lane 1) or BSA preincubated with 150 $\mu$M $Cu^{2+}$ (lane 2). LDL incubated with 150 $\mu$M $Cu^{2+}$ in the presence of regular BSA (lane 3) or BSA pre-incubated with 0.5 mM (lane 4), 2 mM (lane 5), and 10 mM (lane 6) $Zn^{2+}$. C: LDL incubated without $Cu^{2+}$ (lanes 1 and 2) or with 100 $\mu$M $Cu^{2+}$ (lanes 3 and 4) in the presence of 3% nondialyzed (lanes 1 and 3) or dialyzed (lanes 2 and 4) BSA. See legend to Fig. 3 for the additional electronegative band.

or EDTA from bovine plasma from which the albumin was isolated). Alternatively, the removal of certain compounds from the BSA preparation may change the protein conformation and its ability to bind $Cu^{2+}$ ions. The second mechanism seems more likely since our attempts to demonstrate antioxidant properties of the dialysis buffer against which BSA had been dialyzed were unsuccessful.

The above experiments suggest that the LDL protection by albumin against $Cu^{2+}$-mediated modification reflects the capability of BSA to interact with copper ions. By measuring LDL oxidation one can detect two stages of BSA saturation with $Cu^{2+}$. At concentrations below 100 $\mu$M (in 3% BSA solutions) the ions are bound very tightly and are not able to induce the oxidative cascade in the presence of LDL. At higher concentrations the $Cu^{2+}$ ions are bound in such a manner that they can interact with LDL and mediate its oxidation. Our results are consistent with the presence in albumin of the high affinity binding site (38) for copper and nickel, but not zinc ions. The binding of the first one or two copper ions to that high affinity site can be followed by a much weaker binding of several additional ions by electrostatic interactions (40). These two types of copper binding are probably reflected by the two stages of albumin saturation with $Cu^{2+}$ that we observed in our oxidation experiments. Zinc ions at high concentrations can partially saturate the low affinity binding sites but the remaining antioxidant effect of albumin most likely indicates the inability of zinc ions to interact with the high affinity site. Factors affecting albumin conformation in solution can probably modulate its capability to interact with copper ions.

Interestingly, even at high $Cu^{2+}$ concentrations (500 $\mu$M), resulting in a substantial oxidation of LDL, the apoB fragmentation is considerably less advanced in the presence of albumin than in its absence. The lack of effect of LDL oxidation on the immunoreactivity of 4G3 in plasma or in 3% BSA (in contrast to the treatment of isolated LDL) most likely reflects the more limited fragmentation of apoB in the presence of albumin. This is probably due to albumin interactions with LDL.

One may question the physiological relevance of processes occurring in plasma incubated with 500 $\mu$M $Cu^{2+}$. It seems likely, however, that what happens in plasma after overcoming the albumin protection against $Cu^{2+}$-mediated modifications can be brought about under more physiological conditions by different oxidative systems. The observation that in whole plasma the CETP-containing lipoproteins are sensitive to lower concentrations of $Cu^{2+}$ than those required to affect LDL points to some new possible consequences of oxidative processes in vivo.

After this work had been completed, Ohta et al. (41) described the protective effects of albumin and HDL (especially the LpA-I without apoA-II) against the $Cu^{2+}$-mediated oxidation of LDL. The protective effect of HDL could be seen only during the first 6 h of oxidative treatment. After 12 h of incubation with $Cu^{2+}$ the presence of HDL did not affect the degree of LDL oxidation, in agreement with the results of our previous experiments (Zawadzki, Z., R. W. Milne, and Y. L. Marcel, unpublished observation). Therefore, it is unlikely that the presence of HDL could explain the behavior of LDL during the oxidation of plasma in our experiments, in which the $Cu^{2+}$ treatment lasted up to 48 h.

In our ealier experiments (18) the oxidation of isolated LDL always resulted in a simultaneous decrease in its immunoreactivity with 4G3 and the opposite effect with $B_{sol}7$; these were accompanied by an increase in agarose mobility and apoB fragmentation. In the presence of albumin as in whole plasma, only one of these effects, the increased expression of $B_{sol}7$ epitope, could be brought about independently of the others by using the appropriate concentration of $Cu^{2+}$ (50 $\mu$M). In conclusion, the increase in $B_{sol}7$ epitope upon $Cu^{2+}$ treatment, even in the presence of albumin or in whole plasma, remains the most sensitive parameter of LDL apoB oxidative modification. However, under the same conditions, the disappearance of $\alpha_2$-migrating apoA-I and the modification of CETP association with lipoprotein represent even more sensitive parameters for oxidation.

The partially purified CETP was a gift from the laboratory of Dr. Alan Tall. R. W. Milne is a scientist of the Medical Research Council of Canada. This work was supported by a grant (PG-27) from the Medical Research Council of Canada.

*Manuscript received 22 June 1990 and in revised form 5 November 1990.*

## REFERENCES

1. Kita, T., Y. Nagano, M. Yokode, K. Ishii, N. Kume, A. Ooshima, H. Yoshida, and C. Kawai. 1987. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **84:** 5928–5931.
2. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
3. Palinski, W., M. E. Rosenfeld, S. Yla-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum. 1989. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. USA.* **86:** 1372–1376.
4. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
5. Schuh, J., G. F. Fairclough, and R. H. Haschemyer. 1978. Oxygen-mediated heterogeneity of apo-low-density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **75:** 3173–3177.
6. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L.

Witztum, and D. Steinberg. 1985. Essential role of phospholipase $A_2$ activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.
7. Jurgens, G., J. Lang, and H. Esterbauer. 1986. Modification of human low-density lipoprotein by the lipid peroxidation product 4-hydroxynonenal. *Biochim. Biophys. Acta.* **875:** 103–114.
8. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
9. Hiramatsu, K., H. Rosen, J. W. Heinecke, G. Wolfbauer, and A. Chait. 1987. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis.* **7:** 55–60.
10. Fong, L. G., S. Parthasarathy, J. L. Witztum, and D. Steinberg. 1987. Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *J. Lipid Res.* **28:** 1466–1477.
11. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1988. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase $A_2$ mimics cell-mediated oxidative modification. *J. Lipid Res.* **29:** 745–753.
12. Parthasarathy, S., E. Wieland, and D. Steinberg. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **86:** 1046–1050.
13. Hoff, H. F., J. O'Neil, G M. Chisolm III, T. B. Cole, O. Quehenberger, H. Esterbauer, and G. Jurgens. 1989. Modification of low density lipoprotein with 4-hydroxynonenal induces uptake by macrophages. *Arteriosclerosis.* **9:** 538–549.
14. Morel, D. W., P. E. DiCorleto, and G. M. Chisolm. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis.* **4:** 357–364.
15. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
16. Zhang, H., W. B. Davis, X. Chen, R. L. Whisler, and D. G. Cornwell. 1989. Studies on oxidized low density lipoproteins. Controlled oxidation and a prostaglandin artifact. *J. Lipid Res.* **30:** 141–148.
17. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6503.
18. Zawadzki, Z., R. W. Milne, and Y. L. Marcel. 1989. An immunochemical marker of low density lipoprotein oxidation. *J. Lipid Res.* **30:** 885–891.
19. Marcel, Y. L., D. Jewer, L. Leblond, P. K. Weech, and R. W. Milne. 1989. Lipid peroxidation changes the expression of specific epitopes of apolipoprotein A-I. *J. Biol. Chem.* **264:** 19942–19950.
20. Kunitake, S. T., K. J. La Sala, and J. P. Kane. 1985. Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. *J. Lipid Res.* **26:** 549–555.
21. Marcel, Y. L., R. McPherson, M. Hogue, H. Czarrnecka, Z. Zawadzki, P. K. Weech, M. E. Whitlock, A. R. Tall, and R. W. Milne. 1990. Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipemic subjects. *J. Clin. Invest.* **85:** 10–17.
22. Sammett, D., and A. R. Tall. 1985. Mechanisms of enhancement of cholesteryl ester transfer protein activity by lipolysis. *J. Biol. Chem.* **260:** 6687–6697.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
24. Marcel, Y. L., M. Hogue, R. Theolis, and R. W. Milne. 1982. Mapping of antigenic determinants of human apolipoprotein B using monoclonal antibodies against low density lipoproteins. *J. Biol. Chem.* **257:** 13165–13168.
25. Milne, R. W., R. Theolis, R. B. Verdery, and Y. L. Marcel. 1983. Characterization of monoclonal antibodies against human low density lipoprotein. *Arteriosclerosis.* **3:** 23–30.
26. Marcel, Y. L., T. L. Innerarity, C. Spilman, R. W. Mahley, A. A. Protter, and R. W. Milne. 1987. Mapping of human apolipoprotein B antigenic determinants. *Arteriosclerosis.* **7:** 166–175.
27. Marcel, Y. L., M. Hogue, P. K. Weech, and R. W. Milne. 1984. Characterization of antigenic determinants on human solubilized apolipoprotein B. *J. Biol. Chem.* **259:** 6952–6957.
28. Milthorp, P., P. K. Weech, R. W. Milne, and Y. L. Marcel. 1986. Immunochemical characterization of apolipoprotein A-I from normal human plasma. *Arteriosclerosis.* **6:** 285–296.
29. Hesler, C. B., A. R. Tall, T. L. Swenson, P. K. Weech, Y. L. Marcel, and R. W. Milne. 1988. Monoclonal antibodies to the $M_r$ 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* **263:** 5020–5023.
30. Greenwood, F. C., E. M. Hunter, and J. S. Glover. 1963. The preparation of $^{131}$I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89:** 114–123.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
32. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
33. Gambert, P., C. Lallemant, A. Athias, and P. Padieu. 1982. Alterations of HDL cholesterol distribution induced by incubation of human serum. *Biochim. Biophys. Acta.* **713:** 1–9.
34. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242:** 173–181.
35. Spector, A. A., and J. C. Hoak. 1969. An improved method for the addition of long-chain free fatty acid to protein solution. *Anal. Biochem.* **32:** 297–302.
36. Vézina, C. A., R. W. Milne, P. K. Weech, and Y. L. Marcel. 1988. Apolipoprotein distribution in human lipoproteins separated by polyacrylamide gradient gel electrophoresis. *J. Lipid Res.* **29:** 573–585.
37. Frei, B., L. England, and B. N. Ames. 1989. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. USA.* **86:** 6377–6381.
38. Peters, T., Jr. 1975. Serum albumin. *In* The Plasma Proteins. 2nd Ed. Vol. 2. F. W. Putnam, editor. Academic Press, 133–181.
39. Steinbrecher, U. P., and P. H. Pritchard. 1989. Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase. *J. Lipid Res.* **30:** 305–315.
40. Peters, T., Jr. 1970. Serum albumin. *Adv. Clin. Chem.* **13:** 37–111.
41. Ohta, T., K. Takata, S. Horiuchi, Y. Morino, and I. Matsuda. 1989. Protective effect of lipoproteins containing apoprotein A-I on $Cu^{2+}$-catalyzed oxidation of human low density lipoprotein. *FEBS Lett.* **257:** 435–438.