# Oxidation-labile subfraction of human plasma low density lipoprotein isolated by ion-exchange chromatography

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**Abstract** We isolated subfractions of human plasma low density lipoprotein (LDL) using ion-exchange chromatography. Plasma LDL from normolipidemic subjects were applied to a DEAE Sepharose 6B column. After elution of the bulk of LDL at 150 mM NaCl (the major fraction), the residual LDL was eluted at 500 mM NaCl and designated as the minor fraction. The minor fraction, only less than 1% of total LDL, tended to be somewhat similar in certain properties to oxidized LDL, e.g., an increased negative charge, higher protein/cholesterol ratio, and a higher flotation density than native LDL. These results were consistent with data reported by Avogaro et al. (1988. *Arteriosclerosis.* **8:** 79–87). However, assays of $^{125}$I-labeled LDL binding to fibroblasts revealed that the minor fraction possessed binding activity for LDL receptors equal to that of the major fraction. Incorporation of [$^{14}$C]oleate into cholesteryl ester [acyl-CoA:cholesterol acyltransferase (ACAT) activity] in mouse peritoneal macrophages incubated with the minor fraction was only slightly greater than that with the major fraction. Incubation of the minor fraction with 0.5 $\mu$M $Cu^{2+}$ caused a remarkable stimulation of ACAT activity, while stimulation by the major fraction required incubation with 5 $\mu$M $Cu^{2+}$, suggesting that the minor fraction was relatively labile to oxidation. The minor but definite presence of a plasma LDL subfraction more negative and susceptible to oxidation implicates the possibility of its association with atherogenesis. —**Shimano, H., N. Yamada, S. Ishibashi, H. Mokuno, N. Mori, T. Gotoda, K. Harada, Y. Akanuma, T. Murase, Y. Yazaki, and F. Takaku.** Oxidation-labile subfraction of human plasma low density lipoprotein isolated by ion-exchange chromatography. *J. Lipid Res.* 1991. **32:** 763–773.

**Supplementary key words** oxidized LDL • oxidation • atherogenesis

Intense research has been carried out on the atherogenic lipoproteins. A possible atherogenic lipoprotein is oxidized LDL, which is rapidly taken up by cultured macrophages, precursors of the foam cells found in early atherosclerotic lesions (1–5). This modified LDL is formed in vitro by treating native LDL with copper or iron ions or exposing it to cultured cells of major components of atheromatous lesions such as endothelial cells, smooth muscle cells, or macrophages (6–12). Several lines of evidence indicate that cholesteryl ester, a major component in atheromatous lesions, is derived from plasma LDL (13, 14). It is speculated that oxidative modification of LDL in the local artery wall, resulting in enhanced uptake by macrophages through scavenger receptors, causes the formation of foam cells as an initial process in atherogenesis (1). Some observations suggest the presence of oxidatively modified LDL in atherosclerotic lesions (15–17). However, there are some in vivo systems that protect plasma components from oxidation, e.g., vitamin E and cytosolic reduced glutathione (GSH), which would prevent formation of oxidized LDL (9, 18–22). Therefore, it is unlikely that atherogenic LDL is generated in the circulation. Even if it existed in blood, it would soon be recognized by scavenger receptor and rapidly removed through this pathway by the reticuloendothelial system (23, 24). How plasma LDL changes and contributes to atheroma formation is poorly understood. We investigated the possibility of an atherogenic lipoprotein that could exist intrinsically in blood and cause atherosclerosis. Paying special attention to an increase in negative charge, which is characteristic of oxidized LDL, we tried to isolate an LDL subfraction with a greater negative charge than native

Abbreviations: DEAE, diethylaminoethyl; LDL, low density lipoprotein; apo, apolipoprotein; apoB, apolipoprotein B-100; ACAT, acyl-CoA:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; RPMI1640, Roswell Park Memorial Institute's medium 1640; PBS, phosphate-buffered saline; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; BSA, bovine serum albumin; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; TBARS, thiobarbituric acid reactive substances; MDA, malonaldehyde equivalent; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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LDL from plasma LDL by using ion exchange chromatography, and compared this fraction with native LDL in terms of biochemical characteristics. Recently Avogaro, Bittolo Bon, and Cazzolato (25) demonstrated that circulating LDL displays heterogeneity upon DEAE-Sepharose ion-exchange chromatography, and characterized an LDL subfraction with more negative charge, designated modified LDL. In the current study, we have demonstrated that a minor fraction of LDL is similar to modified LDL in some properties and, further, is more susceptible to oxidation than native LDL.

## MATERIALS AND METHODS

### Materials

DEAE-Sepharose 6B was purchased from Pharmacia. [$^{125}$I]Na and [$^{14}$C]oleate were from ICN. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute's medium 1640 (RPMI 1640), phosphate-buffered saline (PBS), and fetal calf serum (FCS) were from Gibco. Bovine serum albumin (BSA), butylated hydroxytoluene (BHT), benzamidine, gentamycin, and Coomassie brilliant blue were from Sigma. Molecular weight standards of proteins and column were from Bio-Rad. Other reagents and materials used were of all first grade. DDY mice and New Zealand White rabbits were from Nippon Bio-supp Center.

### Lipoprotiens and iodination

Blood was taken from nine healthy normolipidemic volunteers fasted more than 14 h. Each plasma sample was freshly obtained and contained 1 mg/ml EDTA, 1 mg/ml $NaN_3$, 10 $\mu$g/ml gentamycin, and 0.3 mg/ml benzamidine. BHT (100 $\mu$M) was also added to plasma in some experiments. LDL was isolated by sequential ultracentrifugation at d 1.019–1.063 g/ml (using KBr to adjust the density) in a Beckman 50.2 rotor at 45,000 rpm at 12°C for 20 h, and recentrifuged at d 1.063 g/ml in a 40.3 rotor (26). LDL was dialyzed against buffer containing 0.1 mg/ml EDTA, 0.1 mg/ml sodium azide, 10 mM Tris-HCl (pH 7.4), and the indicated concentration of NaCl. Iodination of LDL was performed as described by McFarlane with a slight modification (27). Free iodine was removed on Sephadex G 50 (PD10, Pharmacia). Iodinated LDL was dialyzed against 5 l of the buffer containing 150 mM NaCl, 2 mM sodium phosphate (pH 7.4), and 0.1 mg/ml EDTA with four exchanges. The specific activity of iodinated LDL was 200–400 cpm/ng protein. More than 98% and 90% of the radioactivity of iodinated LDL was precipitable with TCA and isopropanol, respectively, and less than 10% was extracted into the chloroform–methanol phase.

### Ion-exchange chromatography

DEAE Sepharose CL6B was used in a 1.5 × 30 cm column (bed volume 50 ml).

### Linear gradient elution

The column was extensively washed with starting buffer containing 10 mM NaCl, 10 mM Tris-HCl, and 0.1 mg/ml EDTA, pH 7.4. Then approximately 30–40 mg of LDL protein dialyzed against the starting buffer was applied to the column. After washing the column with the starting buffer until no protein was detected in the effluent on monitoring at 280 nm (usually with more than 10 bed volumes of the buffer), LDL was eluted with a linear gradient from 10 to 300 mM NaCl at a flow rate of 0.6 ml/min. The effluent was collected in 2-ml fractions and monitored at 280 nm for the detection of proteins and at 254 nm for the detection of fatty acid conjugated dienes.

### Step-wise elution

LDL was applied to the column as described above (Linear gradient elution). The applied LDL was eluted with the buffer containing 150 mM NaCl. After washing the column, the remaining LDL was completely eluted with the buffer containing 500 mM NaCl. Each fraction was monitored at 280 nm and peak fractions were collected as a major fraction for 150 mM NaCl effluent and as a minor fraction for 500 mM NaCl effluent. KBr was added to the major and minor fractions to adjust the density to 1.063 g/ml. Each fraction was ultracentrifuged at 38,000 rpm for 24 h in a 40.3 rotor. The LDL fractions were dialyzed against the buffer containing 150 mM NaCl, 5 mM sodium phosphate, and 0.1 mg/ml EDTA, pH 7.4. The protein and cholesterol concentrations of both fractions were determined immediately as described below, and the major fraction was diluted to the same cholesterol concentration of the minor fraction with the same buffer. Then both fractions were used in the experiments. $^{125}$I-labeled LDL was subjected to the same procedure to obtain labeled major and minor fractions.

### Assays

The protein content in each sample was determined by the method of Lowry et al. (28) with BSA as a standard. Total cholesterol, free cholesterol, triglyceride, and phospholipid in the samples were determined by enzymatic methods (29–31). Lipid peroxides were measured as thiobarbituric acid reactive substances (TBARS) with tetramethoxypropane, which produces malonaldehyde (MDA), as a standard according to Yagi's method (32). Agarose electrophoresis was performed on pol E film (Corning, New York). Electrophoresis was performed in buffer containing 50 mM sodium barbital, 1 mM EDTA, and 0.1% sodium azide, pH 8.6. Lp[a] was measured by a commer-

cial kit using enzyme immunoassay (TintElize Lp[a], Biopool AB, Umeå, Sweden).

### Density gradient ultracentrifugation

The procedure was a modification of one described previously (33). The density was adjusted with NaCl. Three different density solutions, 3.6 ml of d 1.06 g/ml, 4 ml of d 1.03 g/ml, and 3.6 ml of d 1.0 g/ml, were layered sequentially from the bottom to the top in a Beckman centrifuge tube (1.4 × 8.9 cm). The middle portion contained the $^{125}$I-labeled major fraction (1,500,000 cpm) or minor fraction (1,000,000 cpm). The samples were centrifuged in a Beckman SW-41 rotor at 40,000 rpm for 24 h at 16°C. The top fraction (1 ml) was obtained by tube slicing, and then 10 fractions (1 ml/fraction) were collected sequentially from the top by careful aspiration with a pipette. After measuring the radioactivity (A) of each fraction, 50 μl of concentrated LDL solution (2 mg/ml) as carrier and 0.95 ml of isopropanol were added and mixed thoroughly (34). After incubation at room temperature for 1 h, an aliquot of the mixture was taken to measure its radioactivity (B). Then the mixture was centrifuged at 10,000 *g* for 10 min at room temperature and a half volume of the supernatant was taken to measure its radioactivity (C). The radioactivity in apoB was calculated as A × (B − C × 2)/B. The density of each fraction was measured by a conductivity meter, with a standard of NaCl solutions. The mean densities of 11 fractions from the top to bottom were 1.003, 1.005, 1.008, 1.012, 1.018, 1.025, 1.030, 1.036, 1.042, 1.052, and 1.059 g/ml, respectively.

### Electrophoresis

The LDL proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) as described by Laemmli (35). Aliquots of the LDL fractions eluted by chromatography, which contained 50 μg of protein, were delipidated by 20 volumes of ethanol–ethyl ether 2:1 (vol/vol). After centrifugation at 10,000 rpm for 15 min, the pellets were dissolved in 50 μl of 3% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 0.025% Bromophenol blue, and 5% mercaptoethanol, and heated at 100°C for 3 min. Electrophoresis was performed in 3–10% gradient polyacrylamide slab gels containing 1% SDS. After the electrophoresis, the gel was stained with Coomassie brilliant blue or silver (36).

### Immunoblotting

After electrophoresis, the samples were electrophoretically transferred from the gel to nitrocellulose membranes in the buffer containing 20 mM Trisbase, 150 mM glycine, 0.02% SDS, and 20% methanol at 200 mA for 16 h. The sheets were immersed in blocking buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 50 mg/ml BSA for 1 h at room temperature and incubated with the same buffer containing anti-human apolipoprotein B (apoB) goat serum (purchased from Daiichi Pure Chemical, Co., Ltd) for 24 h at 4°C. The sheets were then washed twice for 15 min with the washing buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.1% SDS, 0.2% Nonidet P40, and 0.25% deoxycholate, and incubated in the buffer containing $^{125}$I-labeled Protein A (2000 cpm/ng, 0.5 μg/ml) for 24 h at 4°C. The sheets were washed again, air-dried, and autoradiographed.

### Binding assay of LDL to cultured human fibroblasts

Human skin fibroblasts were obtained from punch biopsies of normal subjects and maintained in monolayer cultures in DMEM with 10% FCS in a humidified $CO_2$ incubator in 5% $CO_2$ at 37°C. Fibroblasts of 5 to 15 passages were used for the experiments. The cells were seeded in 3.5-cm dishes and grown for 5 days in the same medium. After 5 days, when the cells were still subconfluent, the medium was removed and the cells were washed with PBS before the addition of DMEM containing 5 mg protein/ml of lipoprotein-deficient serum (LPDS). After 48 h incubation, binding assays were performed as described by Goldstein, Basu, and Brown (37). All the procedures were performed at 4°C. The cells were cooled on ice at 4°C for 30 min. The medium was changed to 1 ml of ice-cold DMEM containing 10% LPDS, 1.5 μg/ml of $^{125}$I-labeled LDL (sp act 200 cpm/ng) and the indicated cholesterol concentrations of LDL fractions. The dishes were shaken on a rotary shaker at 4°C for 1 h. The cells were then washed rapidly with 3 ml of the buffer containing 50 mM NaCl, 150 mM Tris-HCl (pH 7.4), and 2 mg/ml BSA three times, followed by incubation with 2 ml of the same buffer at 4°C for 10 min. After another washing by the same procedure, the cells were washed with the buffer without BSA twice and dissolved in 1 ml of 0.1 N NaOH. The radioactivities of the lysates were counted with a gamma counter as the cell-associated radioactivity. An aliquot of the lysate was used for protein assay to calculate the amount of LDL bound per cell protein (ng/mg cell protein). The binding activity of each LDL fraction was estimated by competition of $^{125}$I-labeled LDL binding to the cells.

### Preparation of human monocyte-derived macrophages

Human monocyte-derived macrophages were isolated by density gradient centrifugation from blood derived from fasting normolipidemic subjects (38). Thirty-five ml of blood anticoagulated with 10 units/ml of heparin was layered over 15 ml of Metorizoate and Ficoll solution (d 1.077 g/ml, Lymphoprep, NYCOMED AS, Oslo, Norway) and centrifuged at 500 *g* for 40 min at 17°C. The mixed mononuclear cell band was removed by aspiration and the cells were washed twice in RPMI 1640 culture medium. The cells were plated at 3 × $10^5$ monocytes/

16-mm dish in the same medium. After 5 h incubation at 37°C in 5% $CO_2$, nonadherent cells were removed by two washes with serum-free medium. The adherent cells were cultured in fresh medium containing 5% autologous serum. Monocyte-derived macrophages were used within 7–14 days of plating.

### Binding assay of $^{125}$I-labeled LDL to human monocyte-derived macrophages

The binding assay of DEAE fractions of human LDL labeled with $^{125}$I was performed at 37°C. After previous 24 h incubation with 0.5 ml/dish of RPMI medium containing 10% LPDS, the cells were incubated with labeled minor or major fractions (400,000 or 200,000 cpm as apoB/dish) at 37°C for 12 h. After removal of the medium, the cells were subjected to the same procedure as described above (Binding assay of LDL to cultured human fibroblasts) for assay of uptake of LDL. The media were then assayed for degraded LDL according to the method of Goldstein et al. (39). Fifty percent TCA (0.125 ml) was added to the medium collected in a microfuge tube (final concentration 10% TCA) to precipitate undegraded labeled LDL. The mixture was left to stand overnight at 4°C and then centrifuged at 12000 rpm. The supernatant was mixed with a half volume of 10% $AgNO_3$ to precipitate free iodine, centrifuged, and the resulting supernatant was counted as degraded LDL in the form of monoiodide tyrosine.

### Preparation of mouse peritoneal macrophages

Resident mouse peritoneal macrophages from unstimulated female DDY mice (25–30 g) were harvested in PBS and anticoagulated with heparin as described by Edelson and Cohn (40). Ten ml of PBS with 100 U of heparin was injected intraperitoneally into a mouse. The fluid from 20–40 mice (3–6 × $10^6$ cells per mouse) was pooled, and the cells were collected by centrifugation at 500 *g* for 10 min at 4°C and washed with 50 ml of PBS. The cells were resuspended in RPMI 1640 containing 10% fetal calf serum and 100 units/ml of penicillin and 100 μg/ml of streptomycin at a final concentration of 3 × $10^6$ cells per ml. Aliquots (0.5 ml) were dispersed onto 16-mm dishes, then incubated in a humidified $CO_2$ incubator (5% $CO_2$/95% air) at 37°C for 2 h. Each dish was then washed with 1 ml of RPMI twice to remove nonadherent cells. The monolayer cells were incubated with RPMI containing 10% FCS. On the day before the experiment, the medium was changed to RPMI containing 10% LPDS.

### Treatment of LDL with copper ion and assay of incorporation of [$^{14}$C]oleate into cholesteryl ester in mouse peritoneal macrophages

The major and minor fractions of LDL (50–150 μg/ml) containing no EDTA were incubated with the indicated concentration of cupric sulfate in RPMI 1640 at 37°C for 24 h. The macrophages preincubated in RPMI containing 5 mg/ml of LPDS for 24 h were incubated with the major or minor fraction (30 μg protein/ml) and [$^{14}$C]oleate–albumin (0.2 mM, 17000 cpm/nmol) for 12 h to measure the reacylation of cholesterol (41). The cells were washed with PBS twice at 4°C and incubated for 30 min with 0.5 ml of hexane–isopropanol 3:2 (vol/vol) at room temperature to extract lipids. The organic solvent collected (1 ml) was mixed with [$^3$H]cholesteryl oleate as an internal standard and evaporated under nitrogen. The lipid extract was dissolved in chloroform and labeled cholesteryl ester was isolated by thin-layer chromatography on silica gel plate using hexane–diethyl ether–acetic acid 130:40:1.5 and counted in scintillation liquid with a beta scintillation counter.

### Turnover studies of DEAE fractions of LDL in rabbits

New Zealand White (NZW) rabbits were kept in individual cages and fed a normal chow diet. Major and minor fractions of $^{125}$I-labeled LDL were prepared as described under Ion exchange chromatography. The $^{125}$I-labeled minor fraction (1.2 × $10^7$ cpm) was injected into a rabbit as a bolus through a marginal ear vein and blood was taken at the indicated time from the other ear vein. Two days later, injection of the $^{125}$I-labeled major fraction (9 × $10^7$ cpm) was performed in the same way. Plasma was isolated from each blood sample. ApoB radioactivity in each plasma sample was calculated as isopropanol-precipitable radioactivity as described in Density gradient ultracentrifugation (42). Removal rate was calculated from the decay curve of injected apoB radioactivity in each fraction by the method of Matthews (43).

## RESULTS

### Isolation of the minor fraction from LDL by ion-exchange chromatography

We analyzed the linear gradient elution pattern of LDL from a DEAE-Sepharose CL 6B column with a salt concentration of 10–300 mM by scanning at 254 nm and 280 nm. **Fig. 1** shows a representative elution profile. Monitoring at 280 nm showed that the bulk of LDL protein was eluted with a NaCl concentration ranging from 50 to 150 mM and sluggish elution was observed at higher concentration. On the other hand, monitoring at 254 nm showed that a considerable amount of lipid peroxides remained in the column after the peak elution of LDL protein was observed. The ratios of absorbance at 254 nm to that at 280 nm were also plotted and the peak occurred at 147 mM. We determined the concentration of 150 mM NaCl as the boundary line and thereafter we eluted applied LDL step-wise at 150 M and 500 mM NaCl. We designated LDL eluted at 150 mM NaCl as the major fraction and LDL eluted at 500 mM NaCl as the minor

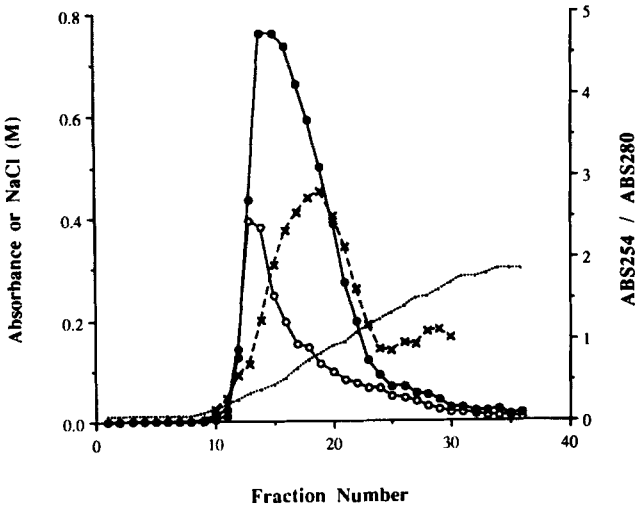


**Fig. 1.** Linear gradient elution profile of LDL from DEAE-Sepharose chromatography. Human plasma LDL was prepared and subjected to ion exchange chromatography (DEAE-Sepharose 6B column) as described in Materials and Methods. The applied LDL was eluted with a linear gradient from 10 mM to 300 mM NaCl (dotted line). The effluent was monitored at 280 nm for the presence of proteins (closed circle) and at 254 nm for that of fatty acid-conjugated dienes as a marker of lipid peroxides (open circle). The ratios of absorbance at 254 nm to that at 280 nm were plotted (cross).

fraction. To confirm the consistency of step-wise elution, we compared linear density gradient elution patterns of native LDL and the minor fraction. We acquired an $^{125}$I-labeled minor fraction from labeled LDL by step-wise elution, mixed this fraction with native LDL, and applied the mixture to the column again. Then linear gradient elution was performed. As shown in **Fig. 2,** the elution peak of the $^{125}$I-labeled minor fraction occurred at more than 150 mM NaCl in the linear gradient elution profile, justifying this method of step-wise elution. It is possible that the minor fraction could be an artificial product formed during isolation steps. To confirm the stability of subfractions and effectiveness of separation, we performed the following two experiments. First, on the assumption that the minor fraction is generated under oxidative change of native LDL, we investigated the effect of BHT (6), a free radical scavenging anti-oxidant, on recovery of minor fraction. BHT (100 $\mu$M) was added immediately after the isolation of plasma to protect LDL from oxidative change during ultracentrifugation, dialysis, or chromatography. However, recovery in protein of the minor fraction was 95.3% (n = 2) of the minor fraction isolated from the same plasma without BHT. Second, we reapplied the major fraction to the DEAE column. If the minor fraction was produced during the column work, we could obtain a considerable quantity of minor fraction from major fraction reapplied to the column as compared with minor fraction acquired initially. However, we obtained less than 20% (19.6%, n = 3) of the initial minor fraction from the second application of the major fraction. These observations suggest that the procedures for isolation are not important in the formation of the minor fraction and that it is present in blood.

### Biochemical properties of the minor fraction

**Fig. 3** shows agarose electrophoresis of the major and minor fractions. The minor fraction exhibited greater mobility to the anodal side than the major fraction, suggesting that the minor fraction has a greater negative charge.

**Table 1** shows the lipid composition of each fraction. We applied 30–40 mg protein of human LDL derived from 200 ml of blood from normolipidemic subjects to the column and acquired 65–380 $\mu$g protein of the minor fraction corresponding to 0.6% of total LDL protein on average (n = 9). The minor fraction was composed of relatively more protein and less cholesterol than the major fraction. No significant differences were observed in the ratio of esterification of cholesterol, composition of triglycerides, and phospholipids. Thiobarbiturate reactive substances (TBARS), a marker of lipid peroxides, in the major and minor fractions were 2.49 and 4.17 nmol MDA/mg protein, respectively. However, both values fell within the normal range of TBARS in LDL reported elsewhere (4–6 nmol MDA/mg protein) (44, 45). To compare the floating densities of the major and minor fractions, density gra-

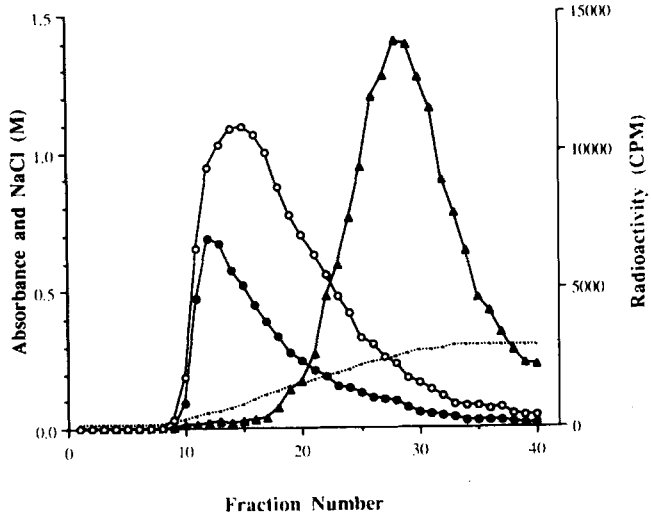


**Fig. 2.** Linear gradient elution profile of $^{125}$I-labeled minor fraction from DEAE-Sepharose chromatography. $^{125}$I-labeled LDL was applied to the DEAE-Sepharose 6B column at 150 mM NaCl and radioactive peak fractions of break-through were collected as the $^{125}$I-labeled major fraction. The column was extensively washed with buffer containing 150 mM NaCl, 0.01% EDTA, and 10 mM Tris-HCl, pH 7.5, and eluted at 500 mM NaCl. Peak fractions of effluent were collected as the $^{125}$I-labeled minor fraction. The $^{125}$I-labeled minor fraction and native LDL were dialyzed against 10 mM NaCl and re-applied to the column, and linear gradient elution at 10–300 mM NaCl (dotted line) was performed as described in Materials and Methods. Absorbance at 280 nm (closed circle), 254 nm (open circle), and radioactivity for the $^{125}$I-labeled minor fraction (closed triangle) were monitored.

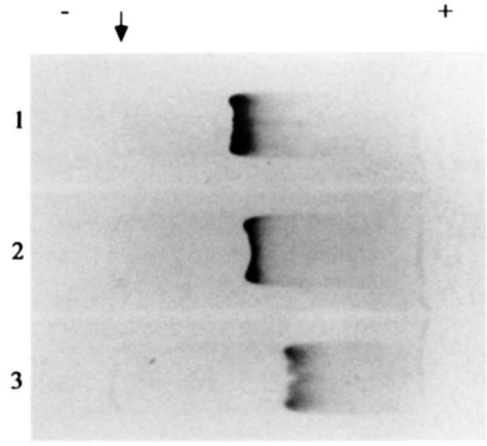


**Fig. 3.** Agarose electrophoresis of the major and minor fractions. Native LDL (1), major (2), and minor (3) fractions of LDL (2 μg protein per lane) were electrophoresed on 0.5% agarose gel (pol E film) and stained with Fat Red 7B. Arrow indicates starting point.

dient ultracentrifugation was performed (**Fig. 4**). The mean densities at which the major and minor fractions floated were 1.030 and 1.036 g/ml, respectively, indicating that the minor fraction has a slightly higher density than the major fraction. We investigated protein moieties of the minor fraction by SDS-PAGE. As shown in **Fig. 5a,** apoB (550 kDa) was detected at the same position as the major fraction. No other obvious extra bands were detected by Coomassie blue. For a more sensitive method of detection, both fractions were compared by silver staining, immunoblot with polyclonal anti-human apoB, and autoradiography of $^{125}$I-labeled fractions (Fig. 5b,c,d). Although several trace bands including apoB-74 and B-26, degradation products of apoB-100, were found in both fractions, no extra bands specific to minor fractions were detected by any method. These data suggest that no gross changes in apoB such as scission products or aggregation occur in the minor fraction. Lipoprotein[a] (Lp[a]) which may be partly isolated in the density range of LDL might involve isolation of the minor fraction (46). Lp[a] was checked in the major and minor fractions from Lp[a]-positive plasma. Lp[a] accounted for 6% protein of the major fraction (3.0 mg/dl Lp[a] in 0.5 mg/ml LDL) but was not detectable in the minor fraction, indicating that Lp[a] is not primarily associated with the minor fraction.

### LDL receptor binding activity of the minor fraction

The two fractions were compared with regard to binding activities to LDL receptors on human fibroblasts. As shown in **Fig. 6,** competition curves of the two fractions against $^{125}$I-labeled native LDL bound to fibroblasts at 4°C coincided completely, suggesting that the minor fraction shows the same binding activity to LDL receptor as the major fraction. Uptake and degradation of iodinated major and minor fractions by human monocyte-derived macrophages were also compared (**Table 2**). No significant differences were observed between the two fractions when the same amount of apoB was added to the assay medium.

To compare the metabolic fate of each fraction in vivo, iodinated major and minor fractions were injected into New Zealand White rabbits sequentially and plasma disappearance of apoB in each fraction was compared (**Fig. 7**). The minor fraction tended to show slightly more rapid initial disappearance as compared with the major fraction. Removal rates of the major and minor fractions were 1.0 LDL pool/day and 1.5 LDL pool/day, respectively. These results suggest that the minor fraction is mainly cleared through pathway(s) similar to native LDL such as LDL receptor and could also be removed through (an)other pathway(s).

### Oxidative modification of the minor fraction with $Cu^{2+}$

Susceptibilities of both fractions to oxidation by $Cu^{2+}$ were estimated by stimulation of incorporation of [$^{14}$C]oleate into cholesteryl ester (ACAT activity) by mouse peritoneal macrophages (**Table 3**). When mouse peritoneal macrophages were incubated with 30 μg protein/ml of the major fraction and 0.2 mM of [$^{14}$C]oleate, synthesis of cholesteryl oleate was 2.3 nmol/mg cell protein per 12 h. Considering that the incorporation without LDL was 1.8 nmol/mg cell protein per 12 h, uptake of the major fraction by human macrophages is much smaller than that by fibroblast because of the small number of LDL receptors expressed on macrophages. While incubation of the major fraction with 0.5 μM of $Cu^{2+}$ caused a minimal increase in ACAT activity, the major fraction incubated with 5 μM of $Cu^{2+}$ stimulated ACAT activity more than 10-fold (27.7 nmol/mg cell protein per 12 h), suggesting that incubation at 5 μM of $Cu^{2+}$ caused the formation of oxidized LDL, which is taken up by scav-

TABLE 1. Lipid compositions of major and minor fractions of LDL

| LDL | Major Fraction | Minor Fraction |
|---|---|---|
| | % | |
| % Total LDL | 99.4 | 0.55 ± 0.25 |
| Cholesteryl ester | 54.6 ± 4.3 | 50.7 ± 4.7 |
| Free cholesterol | 8.3 ± 0.2 | 8.5 ± 0.4 |
| Triglycerides | 3.4 ± 0.7 | 3.7 ± 0.8 |
| Phospholipids | 11.9 ± 6.6 | 12.6 ± 7.0 |
| Protein | 21.9 ± 0.9 | 24.7 ± 1.3 |
| Cholesteryl ester/total cholesterol | 0.79 ± 0.03 | 0.78 ± 0.01 |
| Total cholesterol/protein | 1.96 ± 0.42* | 1.21 ± 0.23* |

LDL prepared from nine different normolipidemic healthy males was used to separate major and minor fractions for assays. Cholesteryl ester was calculated as (total cholesterol − free cholesterol) × 1.68. The values are expressed as means ± SD (% total LDL and total cholesterol/protein; n = 9, other data; n = 3); *, $P < 0.001$.

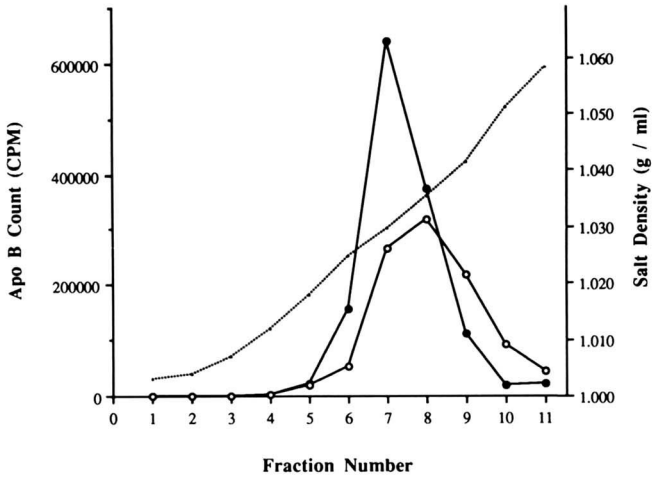


**Fig. 4.** Density gradient ultracentrifugation of $^{125}$I-labeled major and minor fractions. $^{125}$I-labeled LDL subfractions were prepared as described in the Fig. 2 legend. Each fraction was subjected to density gradient ultracentrifugation with the salt density of 1.006–1.063 g/ml, obtained with NaCl, in a SW41 rotor for 24 h. The samples were carefully aspirated into 11 fractions (Fr.1 top, Fr.11 bottom), and the radioactivities of major (closed circle) and minor (open circle) fractions were measured as apoB radioactivity. The recoveries of both fractions as apoB radioactivity were more than 90%. Salt density gradient is shown as the dotted line.

enger receptor on macrophage. These results were the same as those obtained from native LDL. On the other hand, the minor fraction without incubation with $Cu^{2+}$ showed no or only a slight increase in synthesis of esterified cholesterol, 1.0- to 3.0-fold (1.8-fold on average) as compared with the major fraction. The minor fraction incubated with 0.5 $\mu$M $Cu^{2+}$ stimulated ACAT activity up to 12.4 nmol/mg cell protein per 12 h, and this stimulation was not enhanced even at 5 $\mu$M of $Cu^{2+}$. **Fig. 8** shows the patterns of both fractions treated with $Cu^{2+}$ at different concentrations. $Cu^{2+}$ dose-dependent increases in electrophoretic mobility were observed in both fractions. Incubation with 5 or 50 $\mu$M of $Cu^{2+}$ caused a drastic increase in the mobility of the major fraction with a diffuse smear, indicating complete oxidative change; while the minor fraction after incubation with 0.5 $\mu$M and higher concentrations of $Cu^{2+}$ showed the same extent of mobility of a smeared band. The data indicated a marked difference in the susceptibilities of the major and minor fractions to oxidation.

## DISCUSSION

The use of DEAE ion-exchange chromatography has revealed that freshly isolated human LDL has a wide range of heterogeneity in charge as Avogaro et al. reported (25). They observed two peaks in the elution profile on monitoring at 254 nm, and the second peak was collected as modified LDL. However, the elution profile that we observed had only one peak. Therefore, our setting of 150 mM is relative and the major and minor fractions cannot be separated clearly. When the boundary was set at a lower salt concentration, less negative but more minor fraction was isolated. In one experiment, three aliquots of

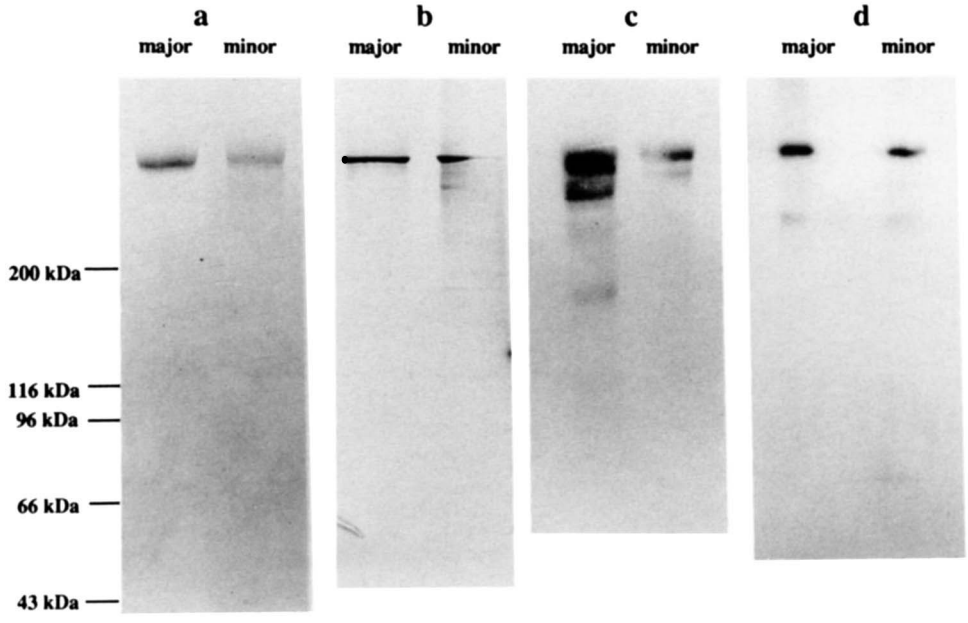


**Fig. 5.** Comparison of apoB in the major and minor fractions. Each fraction (50 $\mu$g protein for Coomassie blue stain, 20 $\mu$g for silver stain, 5 $\mu$g for immunoblot) or each $^{125}$I-labeled fraction (10,000 cpm for autoradiography) was applied to a 3–10% gradient gel. After gradient sodium dodecyl polyacrylamide gel electrophoresis, the gels were stained with Coomassie brilliant blue (a) or silver (b), subjected to immunoblotting using anti-human apoB antibody (c), or autoradiographed (d). Molecular weight standards for a and b are indicated.

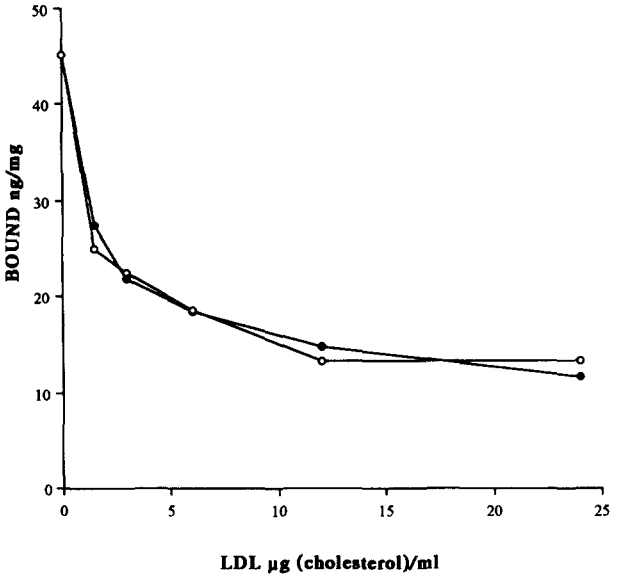


**Fig. 6.** Competition of major (closed circle) and minor (open circle) fractions against $^{125}$I-labeled LDL binding to cultured fibroblasts. Methods for assay of $^{125}$I-labeled LDL binding to cultured fibroblasts were as described by Goldstein et al. (37). Human skin fibroblasts cultured in 3.5-cm dishes with DMEM containing 10% lipoprotein-deficient serum for 48 h and subconfluent cells were used in the experiment. Binding assay was performed at 4°C with 1.5 µg protein/ml of $^{125}$I-labeled LDL (sp act 200 cpm/ng) and the indicated cholesterol concentrations of major or minor fractions of LDL. Cell-associated counts were expressed as means of triplicate assays.

LDL were applied to the columns separately and each major fraction was eluted under the same conditions except at 110, 140, and 150 mM of NaCl, respectively. Each minor fraction was eluted at 500 mM. The mass ratios of each minor fraction versus the respective major fraction eluted at 110, 140, 150 mM, which were calculated from summation of absorbance at 280 nm, were 8.1, 4.2, and 1.6%, respectively. Ratios of absorbance at 254 nm to that at 280 nm in those minor fractions were 7.9, 11.7, and 12.3. To differentiate the characteristics of the two fractions clearly, the boundary was set at 150 mM. The recovery of the minor fraction was very small, which made assays of lipid composition or ACAT activity very difficult. The biochemical characteristics of the minor fraction tended to be similar to those observed in oxidized LDL or LDL isolated from atheroma (1), though the degrees were minimal. However, data from experiments on cell interaction indicate that the changes in chemical characteristics observed in the minor fraction neither abolish LDL binding activity nor cause confirmative recognition by macrophage scavenger receptors. In disagreement with our results, the modified LDL of Avogaro et al. (25) comprised as much as 10% of total LDL. This fraction possessed a decreased esterification ratio of cholesterol, decreased phospholipid, increased conjugated diene products, aggregated apoB, and decreased binding activity to LDL receptors on fibroblasts. These characteristics are considerably different from those of the minor fraction. The reasons for this discrepancy are unknown, but differences in the methods of preparation of LDL and its freshness might be involved.

TABLE 2. Uptake and degradation of major and minor fractions of $^{125}$I-labeled LDL by human monocyte-derived macrophages

| Low Density Lipoprotein | ApoB Radioactivity Added | Major Fraction[a] | Minor Fraction[a] |
|---|---|---|---|
| | *cpm* | | |
| Cell-associated | 200,000 | 11.2 | 23.2 |
| Cell-associated | 400,000 | 30.1 | 31.6 |
| Degraded | 200,000 | 96.6 | 128.1 |
| Degraded | 400,000 | 219.4 | 223.3 |

The indicated apoB radioactivity of the major or minor fraction of $^{125}$I-labeled LDL (sp act = 261 cpm/ng protein) was added to human monocyte-derived macrophages cultured in 0.5 ml of RPMI containing 10% LPDS at 37°C. After 12 h incubation, cell-associated and degraded LDL were measured as described in Materials and Methods. The percentage of apoB in labeled major and minor fractions was 96% and 87%, respectively.

[a]The values are expressed as means of triplicate assays (ng LDL protein/mg cell protein).

The minor fraction exhibited a slightly greater stimulation of ACAT activity than native LDL or the major frac-

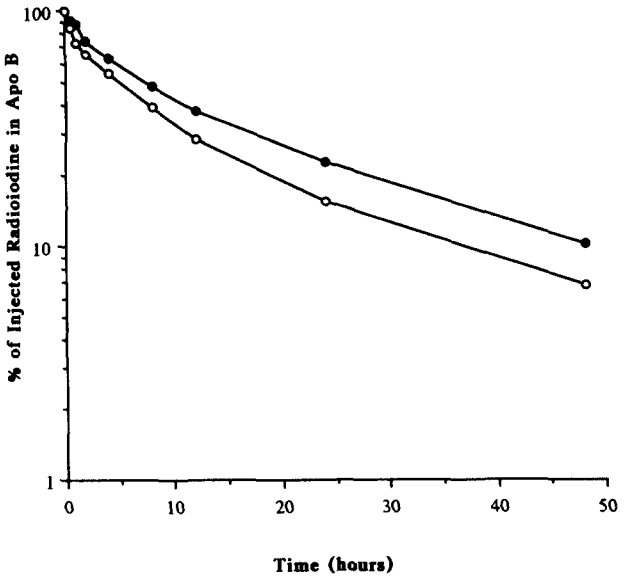


**Fig. 7.** Plasma disappearance of major (closed circle) and minor (open circle) fractions of $^{125}$I-labeled LDL in rabbits. The $^{125}$I-labeled minor fraction ($1.2 \times 10^7$ cpm) and the $^{125}$I-labeled major fraction ($9 \times 10^7$ cpm) were injected into NZW rabbits sequentially at 48-h intervals and the plasma disappearance of apoB radioactivities was measured at the indicated hours. The percentage of apoB counts of major and minor fractions was 95% and 86%, respectively. The residual plasma radioactivity 48 h later after the injection of the $^{125}$I-labeled minor fraction was less than 1% of the initial plasma radioactivity after injection of the $^{125}$I-labeled major fraction. The values are expressed as % of injected radioiodine in plasma apoB after injection in duplicate assay in two independent experiments.

TABLE 3. Cholesterol esterification ([$^{14}$C]oleate→cholesteryl[$^{14}$C]oleate) in mouse peritoneal macrophages incubated with major or minor fraction treated with $Cu^{2+}$

| $Cu^{2+}$ | Major Fraction | Minor Fraction |
|---|---|---|
| $\mu M$ | *nmol/mg cell protein per 12 h* | |
| 0 | 2.3 ± 1.0 | 4.1 ± 3.0 |
| 0.5 | 3.5 ± 0.4 | 12.4 ± 3.1 |
| 5 | 21.8 ± 12.9 | 12.3 ± 2.8 |

Mouse peritoneal macrophages were incubated for 12 h with 0.2 mM [$^{14}$C]oleate and the major or minor fraction (30 $\mu$g/ml) was pretreated with the indicated concentration of copper ion. Measurement of cholesterol esterification was as described in Materials and Methods. The values are means ± SD (nmol/mg cell protein per 12 h) from three independent experiments. The blank value (no LDL) was 1.8 ± 0.4 nmol/mg cell protein per 12 h.

tion. It is unlikely that the minor fraction is taken up through scavenger receptor(s) because apoB in the minor fraction is apparently intact as judged by SDS PAGE, although a minor change cannot be excluded. Interestingly, remarkable stimulation of ACAT activity was observed at lower $Cu^{2+}$ concentration (0.5 $\mu$M) than at the concentration of 5 $\mu$M, which caused (and is reported to cause) formation of oxidized LDL and marked stimulation of ACAT activity in macrophages in native LDL (22). The results of agarose electrophoresis of both fractions treated with $Cu^{2+}$ are consistent with these data. Critical changes of apoB in the major fraction, such as breakdown of the binding domain to LDL receptor and formation of ligand domains for scavenger receptor(s), would take place during incubation between 2 and 5 $\mu$M $Cu^{2+}$, while those in the minor fraction would occur between 0.2 and 0.5 $\mu$M. This suggests that the minor fraction is more susceptible to oxidation by copper ion or is in the pre-oxidative state, although it retains an affinity equivalent to LDL receptors as native LDL. This observation could account for the slight increase in ACAT activity with the minor fraction without $Cu^{2+}$ incubation. During a 12-h incubation, only the minor fraction might be partially oxidized by interaction with macrophages.

The moiety contributing to the negative charge of the minor fraction remains to be determined. Changes in amino acid compositions of apoB or heterogeneity of the content of sialic acid or phospholipid composition in plasma LDL could cause the increased negative surface charge in the minor fraction.

The present study suggests that the minor fraction is generated in blood from native LDL, that it stays in the circulation as long as native LDL, and that it can be cleared mainly through the LDL receptor pathway. However, it might come to another fate as atherogenic LDL. The minor fraction might be transferred to the artery wall, possibly through transendocytosis by endothelial cells, and because of its susceptibility to oxidation, could be preferentially changed into oxidized LDL in sequestered interstitial space in the artery wall, as compared to native LDL, to form an initial atheromatous lesion. It is very important that this pre-oxidized LDL exists in blood, although it is present in a very small quantity. Even if the minor fraction is less than 1% of plasma LDL, it is possible that this small quantity of atherogenic LDL would accelerate atherogenesis over a long interval. To confirm that the minor fraction is atherogenic lipoprotein which intrinsically exists in blood, further investigations are necessary concerning its interaction with endothelial cells or its susceptibility to oxidation under the anoxic state, such as in the artery wall. It would also be very intriguing to known how much minor fraction exists in the plasma of patients with hypercholesterolemia or advanced atherosclerotic lesions. The established effects of probucol on the

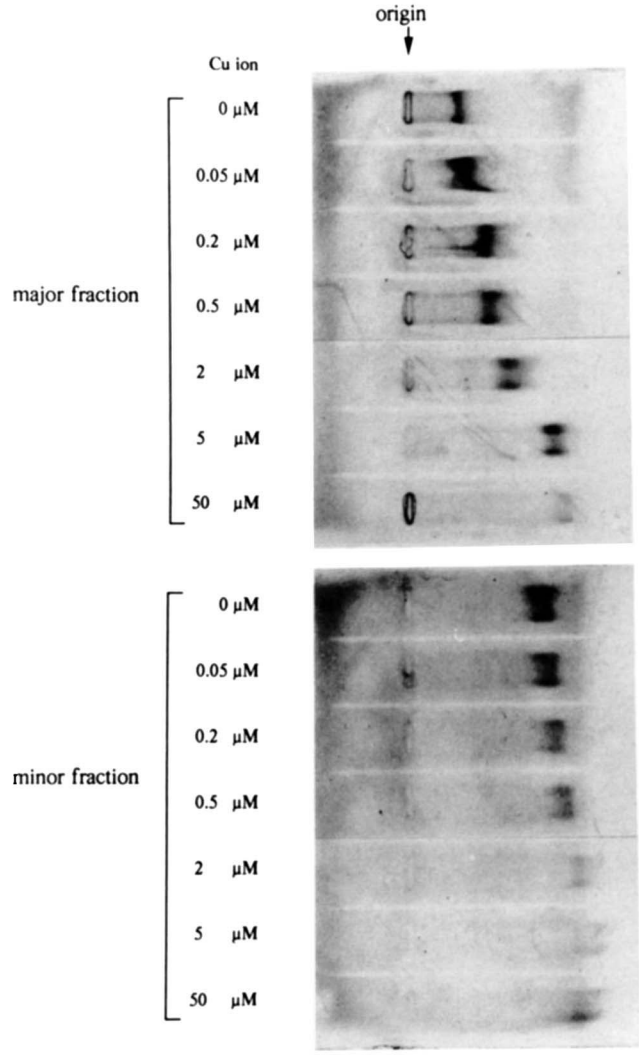


**Fig. 8.** Agarose electrophoresis of the major and minor fractions treated with $Cu^{2+}$. Major and minor fractions (200 $\mu$g protein/ml) were incubated in PBS with the indicated concentration of cupric sulfate at 37°C for 24 h, and 5 $\mu$l of each sample was subjected to agarose electrophoresis. The gel was stained with Fat Red 7B.

progression of atherosclerosis in Watanabe Heritable Hyperlipidemic rabbits might be via suppressed production of the minor fraction leading to prevention of the formation of oxidized LDL in local lesions (47, 48).

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