Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins

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Abstract The ability to preserve low density lipoprotein (LDL) preparations frozen for weeks and months without changes in structure or biological properties is of potential use in long-term comparative studies of LDL. We demonstrate that freeze-thawing of LDL causes marked alterations in its structure and biological behavior, and that such changes can be prevented by the addition of sucrose to the LDL solution prior to freezing. Freezing LDL at −70°C in the absence of sucrose resulted in aggregation and fusion of particles as measured by electron microscopy, spectrophotometric absorption, and column gel filtration. This was associated with increased binding affinity of monoclonal antibodies at epitopes distant from the receptor binding region. Functional changes induced by freezing included 3- to 10-fold increases in binding at 4°C and 37°C, and uptake of LDL in fibroblasts, attributable mainly to increases in nonspecific binding processes. Cryopreservation of LDL in 10% sucrose (w/v) completely prevented the structural and functional changes incurred after short-term freezing, and LDL cryopreserved in sucrose for as long as 18 months displayed cell binding, uptake, and degradation very similar to that of freshly obtained LDL.—**Rumsey, S. C., N. F. Galeano, Y. Arad, and R. J. Deckelbaum.** Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins. *J. Lipid Res.* 1992. **33:** 1551–1561.

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A significant problem in the study of low density lipoprotein (LDL) structure and function is the inability to store LDL preparations, unmodified, for long periods of time. LDL undergoes degradative and oxidative damage over the course of a few days to weeks when stored at 4°C (1–4). Although precautions can be taken to minimize alterations in LDL (5), unpredictable modifications can limit the accuracy of comparative studies of LDL that are performed over long time periods (such as in drug or diet trials). Furthermore, some experimental systems used to study LDL, such as cell culture, can show greater variability between experiments than the expected treatment effects on LDL. Consequently, when comparing properties of LDL specimens collected at different times, it would be optimal to store LDL samples in a way that would prevent their modification and thus enable one to simultaneously examine LDL obtained at different times, from single or multiple subjects.

Freezing is widely used to preserve biological samples. However, it has been known for many years that plasma lipoproteins are damaged by freeze-thawing. Cohn, in the 1940s, initially described that "for certain lipoproteins...freezing appears to weaken the attachment of lipid and protein" (6). Although total cholesterol and triglyceride concentrations of sera do not appear to be affected by freezing (7), changes have been found in individual lipoprotein fractions. Storage of human serum at −20°C for 27 weeks resulted in significant alterations in lipid concentrations of isolated lipoprotein classes (8). Freezing plasma to −28°C for as short a period as 7 days caused a decrease in lipoprotein yield upon ultracentrifugation (9), and freezing plasma to −70°C resulted in a significant decrease in apoB content as measured by radial immunodiffusion (10).

In 1957 Lovelock (11) reported that addition of sucrose to β-lipoproteins prior to freezing can help prevent freeze-related loss of solubility, but this approach has not been applied to cryopreservation of lipoprotein particles in other settings. In this paper we report our investigation of the ability of sucrose to prevent freezing-induced damage of human LDL. We found that freezing of LDL to −70°C, with subsequent thawing, resulted in marked alterations in LDL structural and cell-binding characteristics. The addition of 10% sucrose (w/v) prior to freezing prevented such changes and maintained LDL with physi-

Abbreviations: LDL, low density lipoprotein; Mab monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetate; TCA, trichloroacetic acid; DMEM, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum; TBAR, thiobarbituric acid-reacting substances; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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cal, chemical, and biological properties similar to those of freshly isolated LDL, even after 18 months of storage.

MATERIALS

Sodium ^{125}I was purchased from Amersham (Arlington Heights, IL). Bovine serum albumin (BSA) (A-2153, lot 68F-0469), sucrose, N-2-hydroxyethylpiperzine-N-2-ethanesulfonic acid (HEPES), disodium EDTA, malondialdehyde, sodium dodecyl sulfate, Tris-base, and glycerol were obtained from Sigma (St. Louis, MO). Falcon tissue culture plates (12 wells/plate) were obtained from Becton Dickinson (Oxnard, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Hyclone Laboratories (Logan, UT). L-Glutamine (200 mM), penicillin (10,000 U/ml), and streptomycin (10,000 μg/ml) were obtained from Hazelton (St. Lenexa, KS). Thiobarbituric acid obtained from J. T. Baker (Phillipsburg, NJ), bromophenol blue from LKB (Gaithersburg, MD), and β-mercaptoethanol from Bio-Rad (Richmond, CA) were also used.

METHODS

Low density lipoproteins

Blood from normolipidemic subjects was collected into Vacutainer tubes containing EDTA and the plasma was separated by low speed centrifugation. Disodium EDTA (1.2 g/l), NaN_3 (0.1 g/l), and aprotinin (10,000 U/l) were added to the plasma (12) and LDL was isolated by sequential ultracentrifugation (1.019 g/ml < d < 1.063 g/ml) (13). LDL was radioiodinated using the procedure of Bilheimer, Eisenberg, and Levy (14). The specific activity of ^{125}I-labeled LDL ranged between 100 and 400 cpm/ng of LDL protein. Over 99% of the radioactivity was precipitable after a 60-min incubation with 10% (v/v) trichloroacetic acid at 4°C. LDL preparations were dialyzed against saline (150 mM NaCl, 0.24 mM EDTA, pH 7.4) after ultracentrifugation, iodination, and freeze–thawing.

Freezing

LDL solutions (1–1.5 ml aliquots) were frozen to −70°C in 1.5-ml plastic microcentrifuge tubes by placement into a Fisher 212Z upright −70°C freezer. Samples remained frozen for a minimum of 20 h and a maximum of 18 months. Freezing rate was not controlled but was measured using a temperature probe and found to be about −4°C per min. LDL was thawed by immersion in a 37°C water bath for 3 min.

Sucrose addition

Sucrose was added to LDL solutions by direct addition of a stock sucrose solution (50% sucrose, 150 mM NaCl, 0.24 mM EDTA, pH 7.4). Prior to all experimental procedures, sucrose was removed by dialysis at 4°C against saline (150 mM NaCl, 0.24 mM EDTA, pH 7.4). Dialysis buffer (2000 × sample volume) was changed 3 times over an 24-h period. Using [^3H]sucrose we demonstrated that 88% and 99.96% of the sucrose had been removed from the LDL sample by 6 and 24 h, respectively.

Cells

Human fibroblasts (N1) from the foreskin of normal newborns were plated from frozen stock (6th–12th passage) at a density of 2.7×10^4 cells/well in 12-well plates. Using established methods (12), cells were grown for 5 days in DMEM supplemented with 1% (v/v) glutamine, 1% (v/v) penicillin, 1% (v/v) streptomycin, and 10% (v/v) fetal bovine serum with one change of medium at day 3. At day 5 of culture, cell monolayers were washed once with phosphate-buffered saline (PBS) and further grown for 48 h in the same medium as described above except that fetal bovine serum was replaced with 10% fetal bovine lipoprotein-deficient serum (LPDS) prepared by ultracentrifugation at d > 1.25 g/ml. All experiments were performed on day 7 of culture on confluent fibroblast monolayers.

LDL binding, uptake and degradation

Radioiodinated LDL (^{125}I-labeled LDL) samples were either maintained at 4°C or frozen with or without added sucrose (10% w/v). Binding of ^{125}I-labeled LDL to fibroblasts was measured after a 2.5-h incubation at 4°C in DMEM containing 25 mM HEPES, 1% albumin (w/v), pH 7.4. Nonspecific binding was measured by the addition of 30-fold excess of nonradioiodinated LDL. Specific binding was obtained by subtracting nonspecific bound from total bound ^{125}I labeled LDL.

Binding, uptake, and degradation of ^{125}I-labeled LDL to fibroblasts was measured after a 5.5-h incubation at 37°C in DMEM supplemented with 10% LPDS (12). The medium was removed and the cell monolayers were washed (3×) with PBS containing 0.2% BSA and PBS alone (2×). Cell surface-bound LDL was determined as the amount of radioactivity displaced from the cells after a 60-min incubation at 4°C in a buffer containing 50 mM NaCl, 10 mM HEPES, 4 mg/ml dextran sulfate. LDL uptake was measured as the amount of radioactivity remaining associated with the cells after incubation with dextran sulfate. LDL degradation was calculated as the difference between the amount of non-iodine, non-lipid TCA-soluble radioactivity in the medium and the amount due to spontaneous degradation (measured in parallel incubations with no cells present in the system) (12). As at 4°C, in 37°C experiments, nonspecific uptake and degradation were determined in the presence of 30-fold excess nonradiolabeled LDL.

Competitive monoclonal antibody radioimmunoassays of LDL apolipoprotein B

(Kindly performed by the laboratory of Drs. Ross Milne and Yves Marcel, Institute of Research, Montreal.) The radioimmunoassay with different anti apoB monoclonal antibodies (Mab) was slightly modified from that described previously (15). Immulon II Removawells (Dynatech Laboratories, Chantilly VA) were coated by an overnight incubation with 200 μl of reference LDL (30 μg/ml in 5 mM glycine, pH 9.2) and subsequently saturated by incubation for 1 h with 250 μl of 1% BSA-PBS, pH 7.4. Serial dilutions (150 μl) of test and control LDL were prepared in microtitre plates. One hundred fifty μl of Mab, appropriately diluted in 1% BSA-PBS, was added to the diluted LDL and allowed to incubate for 4 h at room temperature. Aliquots (200 μl) of the LDL-Mab mixture were transferred to the LDL-coated Removawells that had been washed with a solution of 0.15 M NaCl containing 0.025% Tween 20. The wells were incubated overnight and again washed with the Tween-saline solution as above. Two hundred μl of ^{125}I-labeled rabbit anti-mouse IgG (16) (1–2 $\times$ 10^4 cpm/ng) diluted to 83 ng/ml was added to each well and incubated overnight. The wells were washed with the Tween-saline solution as above and counted for bound radioactivity.

Physical analyses

Spectrophotometric measurements were performed on a Beckman DU-50 Spectrophotometer in Fisher polystyrene semi-micro cuvettes.

Gel filtration was performed on a Pharmacia column (40 cm $\times$ 1.6 cm) containing Sepharose CL2B with a flow rate of 0.8 ml/min. The eluent consisted of saline (150 mM NaCl, 0.24 mM EDTA, 0.02% NaN_3). Samples were applied to the column in 500 μl aliquots and collected in 1.8-ml fractions. Prior to column application, all LDL preparations were adjusted to a concentration of 2 mg/ml.

Electron microscopy of LDL particles was performed on a JEOL 1200 EX (Peabody, MA), with negative staining using a modification of a previously described method (17). Briefly, LDL (1 mg/ml) was dialyzed against saline buffer (described above) and 1 drop of sample was placed on a 200-mesh Formvar–carbon-coated grid (Ladd Research Industries, Burlington VT). Excess sample was removed with filter paper, one drop of 2% sodium phosphotungstic acid (pH 7.2) was placed on the grid, and the preparation was air-dried for 30 min. Particle size histograms were generated by measuring the diameter of 200 free standing particles in enlarged photographs using a Peak Scale Lupe (Electron Microscopy Sciences, Ft. Washington, PA).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4% homogeneous and 2.5–16% gradient acrylamide gels (Isolab, Akron, OH). To prepare LDL samples for gel application, LDL was added to an equivalent volume of a gel incubation buffer (1% sodium dodecyl sulfate, 0.1% Tris-base, 20% glycerol, 0.01% bromophenol blue, pH 6.8). β-Mercaptoethanol (20 μl/ml) was added and samples were heated at 100°C for 4 min. Four percent gels were run at 11 amp for 3 h in a 2050 Midget (LKB, Sweden), and 2.5–16% gels were run at 30 amp for 18 h in a GE-2/4 (Pharmacia, Alameda, CA), both powered by an EPS 250/200 (Pharmacia).

To determine susceptibility to oxidation, LDL (1 mg/ml) was oxidatively modified with 2.5 μM $CuSO_4$ in PBS at 37°C for 0–5 h using methods described previously (18). Lipid peroxide content of LDL was determined using a modification of the thiobarbituric acid-reacting substances (TBARS) (19). LDL (100 μg protein) was mixed with 1.5 ml of 0.67% thiobarbituric acid and 1.5 ml of 20% TCA. After heating at 100°C for 30 min, the reaction product was assayed fluorometrically using an Aminco-Bowman spectrophotofluorometer (Silver Spring, MD) with excitation at 515 nm and emission at 553 nm. Pure malondialdehyde was used as a standard.

Far UV circular dichroic spectroscopy was kindly performed by Dr. Mary Walsh, Boston University School of Medicine. Circular dichroism (CD) spectra were recorded on an AVIV 60DS CD spectropolarimeter (AVIV Associates, Inc., Lakewood, NJ) calibrated from 500 to 190 nm with *d*-10-camphorsulfonic acid (1 mg/ml in ethanol). LDL samples were placed in 0.02-cm quartz cells at a concentration of 0.3–0.6 mg protein/ml. Ten spectra for each sample were recorded, averaged, and corrected for baseline contribution due to buffer (20). Molar ellipticity values, [Θ], were calculated and spectra were analyzed by computer for α-helix, β-sheet, β-turn and random coil, at 1-nm intervals between 250 and 190 nm as described previously (21).

Chemical analysis

LDL and cell protein were measured using the method of Lowry et al. (22). LDL triglycerides, total and free cholesterol were analyzed using Boehringer Mannheim enzymatic kits #877557, 290319, and 310328, respectively. LDL phospholipids were analyzed using the Bartlett phospholipid assay (23).

RESULTS

Physical studies

Freezing and subsequent thawing of low density lipoprotein solutions (2 mg/ml) caused a clearly discernable increase in visual opacity; turbidity, measured as "apparent" absorbance at 650 nm, increased further to a maximum after two to four repeated freeze–thawing cycles. The addition of sucrose (10% w/v) maintained LDL with normal

"apparent" absorbance even after repeated freeze-thaw cycles (data not shown). Spectrophotometric analysis of LDL before and after freeze-thawing (325-900 nm) showed a large increase of "apparent" absorbance and alterations in the spectra at all wavelengths tested (**Fig. 1A**). The turbid LDL solution produced after freezing and thawing showed two peaks when studied by gel filtration on a Sepharose CL2B column (Fig. 1B). When the pooled fractions of the early (26-36 ml) and late (46-72 ml) peaks were analyzed spectrophotometrically, the early peak displayed marked turbidity and increased "apparent" absorbance at all wavelengths, whereas the second peak showed an absorbance pattern similar to fresh, unfrozen LDL (control LDL) (data not shown). When sucrose was added to the LDL solution at a concentration of 10% (w/v) prior to freezing, both spectrophotometric absorbance and gel filtration characteristics remained identical to those of unfrozen LDL (Fig. 1 A, B). Normal patterns of absorbance and gel filtration were maintained in LDL cryopreserved with 10% sucrose (w/v) even when it was subjected to six separate freeze-thaw cycles (data not shown).

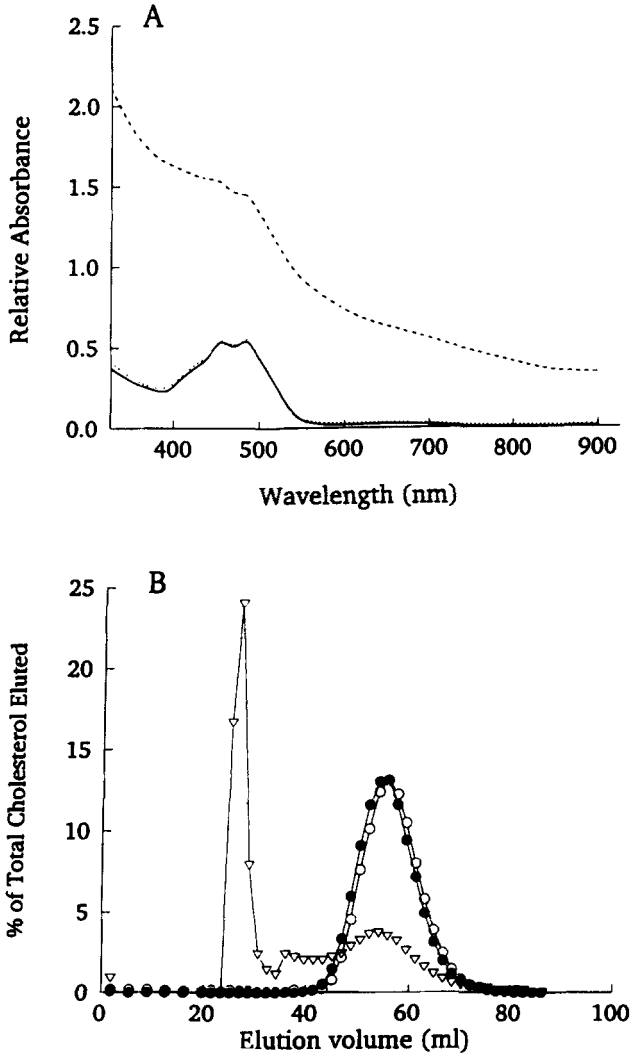


Fig. 1. Spectrophotometric analysis and gel elution profiles of LDL after freeze-thawing. LDL (2 mg protein/ml) was subjected to short-term freeze- thawing (20 h). A) Spectrophotometric absorption over wavelengths of 325-900 nm for LDL maintained at 4°C (—), frozen to −70°C (---), and frozen to −70°C in the presence of added 10% sucrose (w/v) (····). B) After spectrophotometric analysis, the same LDL preparations were applied in 500-μl aliquots to a Sepharose CL2B gel column. The cholesterol content of each 1.8-ml eluent fraction is expressed as a percent of the sum of total cholesterol eluting from the all fractions; LDL maintained at 4°C (●); LDL frozen to −70°C with (○) and without (▽) 10% sucrose (w/v).

The size distribution of LDL particles maintained at 4°C, or frozen to −70°C for 20 h with and without sucrose were evaluated by electron microscopy (**Fig. 2**). LDL frozen without sucrose displayed particle aggregates (Fig. 2B, large arrow), clumping and fusion (small arrow). LDL frozen in the presence of 10% sucrose showed the same morphological characteristics as normal fresh, unfrozen LDL (Fig. 2 A,C). The clumping and aggregation seen in Fig. 2B was not the result of overcrowding, and was not seen in whole-field views of either control LDL or LDL frozen with sucrose. The distribution of LDL particle sizes was relatively homogeneous and almost identical for LDL maintained at 4°C and LDL cryopreserved in sucrose (mean diameter = 20.1 nm ± 2.1 vs. 19.7 nm ± 1.8), while LDL frozen without sucrose showed a bimodal distribution (mean diameter = 26.0 nm ± 7.2) with greater than 40% of particles having a diameter larger than 24 nm. (Fig. 2 D-F). (Because only free-standing particles were measured, the degree of aggregation and fusion was most likely substantially underestimated in samples frozen without sucrose.

LDL lipid-protein composition (protein, phospholipid, triglyceride, cholesteryl ester, and free cholesterol) showed no changes from LDL stored at 4°C, or after freezing to −70°C with sucrose.

We questioned whether the concentration of LDL would influence freeze-thaw-associated changes. Spectrophotometric absorption and gel filtration of LDL frozen at varying concentrations (1-16 mg protein/ml) showed a progressive decrease in the proportion of aggregated LDL as LDL concentration increased (**Fig. 3A**). The addition of 10% sucrose to LDL prior to freezing completely inhibited freeze-thawing-induced aggregation at all concentrations as measured by gel filtration (Fig. 3B).

When increasing concentrations of sucrose were added to 2 mg/ml of LDL, a concentration of LDL at which freeze aggregation was high, there was a dose-dependent decrease in aggregation due to freezing (**Fig. 4**). As measured by gel filtration, the degree of aggregation decreased from approximately 60% of LDL particles without added sucrose to 0% at 5 mg/ml. Higher sucrose concentrations of up to 300 mg/ml (30% w/v) had similar

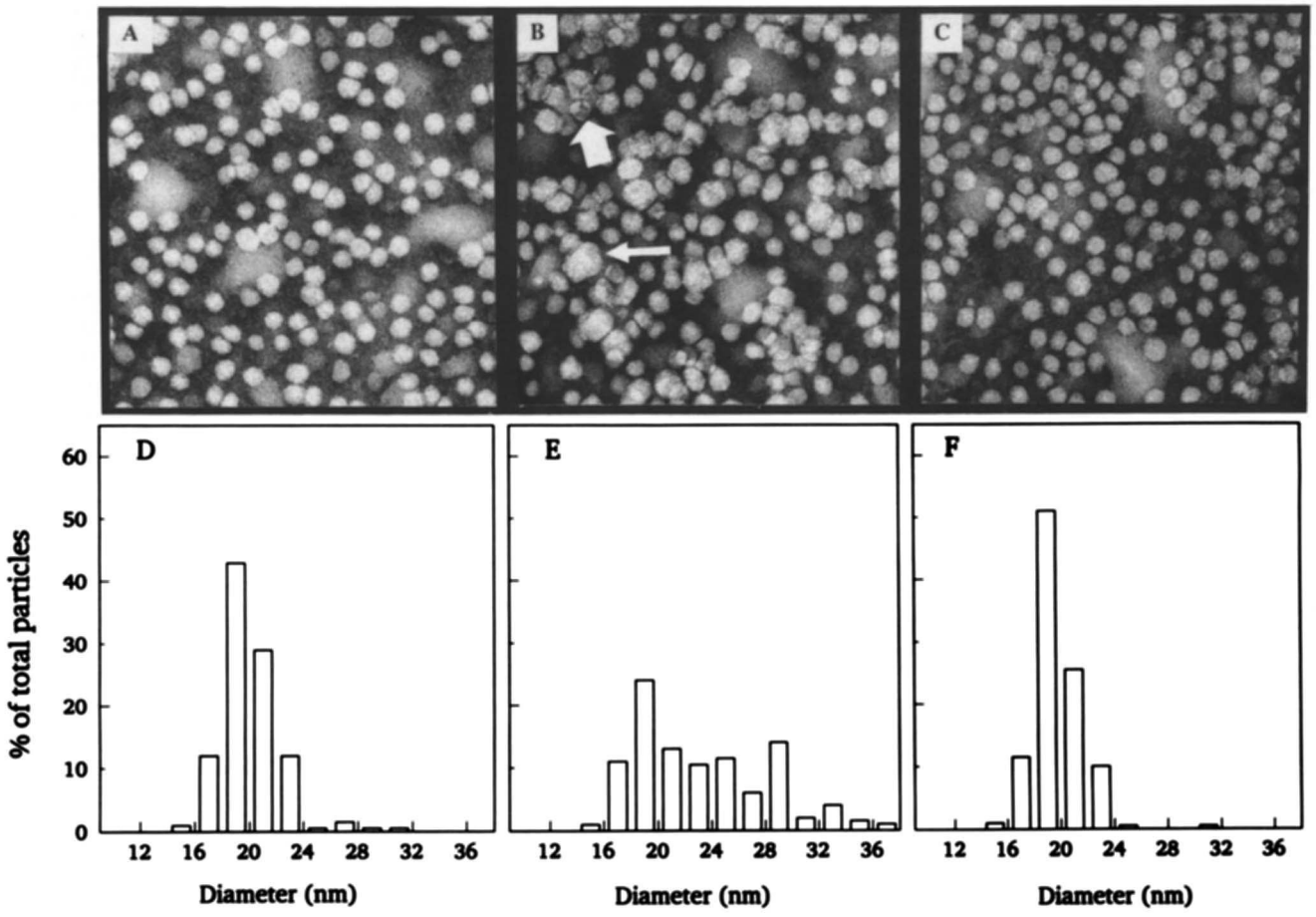


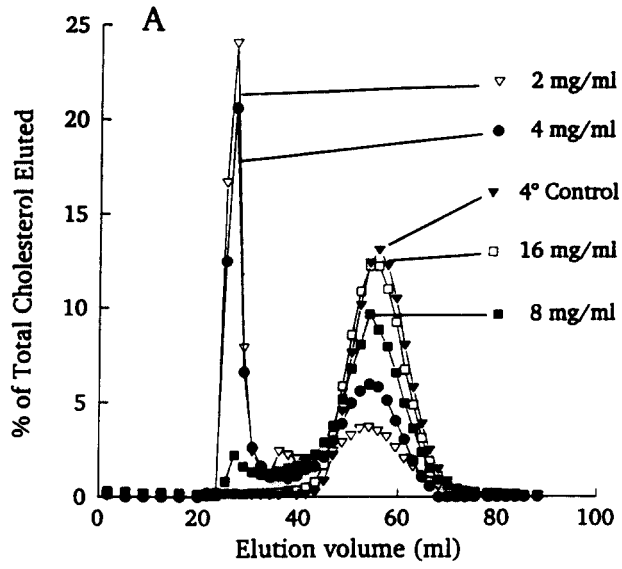
Fig. 2. Electron photomicrographs of negatively stained low density lipoproteins (A–C). Initial instrument magnification was ×80,000. An LDL preparation was divided into three fractions which were either (A) maintained at 4°C, or frozen to −70°C without (B) or with (C) 10% sucrose (w/v). Sucrose was removed from the LDL solution by dialysis prior to electron microscopy. Examples of particle aggregation (large arrow) and fusion (small arrow) can be seen in panel 2B. Frequency distribution of the diameter of 200 particles of each sample is shown below in 2D, E, and F, respectively. Due to marked particle aggregation in the LDL sample frozen without sucrose (2E), only free-standing individual particles were measured.

protective effects (data not shown). We originally chose a 10% sucrose concentration based on previous reports (11). While these experiments suggested that as little as 0.5% may provide adequate short-term freeze–thaw protection for 2 mg of LDL, we continued using 10% sucrose (100 mg/ml) throughout subsequent experiments in order to ensure adequate freeze–thaw protection for long-term freezing for which we still had no data.

LDL samples frozen at −70°C short-term (20 h) or long-term (>18 months) were analyzed for actual or potential oxidative changes by measuring malondialdehyde equivalents and their susceptibility to Cu^{2+} oxidation. No differences were found as compared to control, untreated LDL.

It has been demonstrated that circular dichroism can be used to demonstrate alterations in LDL apoB conformation such as those brought about by changes in LDL particle size (24). Analysis of LDL apoB secondary structure using circular dichroism showed no significant differences between LDL frozen with sucrose and that maintained at 4°C (**Fig. 5**). Turbidity of the LDL solution prevented circular dichroic analysis of LDL frozen in the absence of sucrose.

To assess more specifically regions in LDL apoB where changes due to freeze–thawing might occur, monoclonal antibody studies were performed. The binding of monoclonal antibodies (3A10, 3F5, 4G3, 1D1, and 2D8) to control LDL (maintained at 4°C) and to LDL frozen with or without sucrose was compared. Antibodies 1D1 and 2D8, which recognize epitopes on apoB remote from the LDL receptor-binding domain [amino acid residues 401–582, and 1297–1480, respectively (25)], showed marked increases in binding to freeze–thawed LDL (**Fig. 6**). Our results confirm recent observations that frozen storage of LDL without cryopreservation alters apoB immunoreactivity (26). In contrast, LDL frozen with sucrose dis-



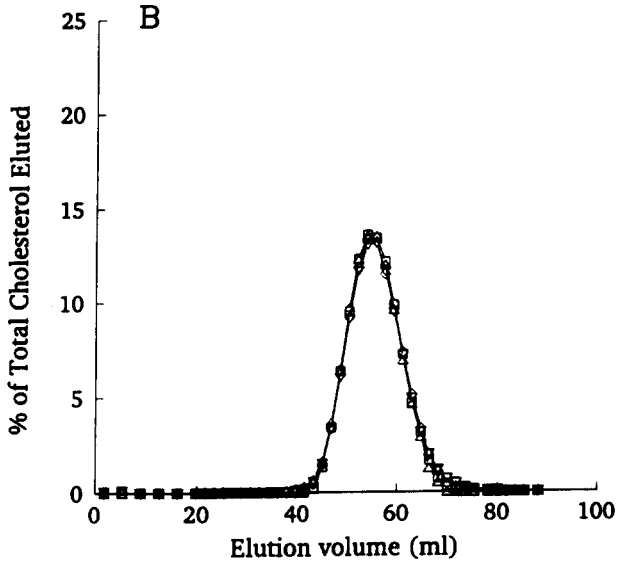


Fig. 3. Effect of LDL concentration on freeze-thaw aggregation. LDL samples of varying concentrations (2–16 mg/ml) were frozen in 1-ml aliquots to −70°C with (B) or without (A) added cryopreservative (10% sucrose (w/v)). Samples were thawed and adjusted to the same final concentration; Sepharose CL2B gel elution profiles were obtained as described in Fig. 1. (Symbols in 3B were changed for clarity, 2 mg/ml (▽), 4 mg/ml (○), 8 mg/ml (□), 16 mg/ml (△).) Data for LDL frozen at 2 mg/ml is the same as that in Fig. 2.

played immunoreactivity for these antibodies similar to that of LDL maintained at 4°C. Antibodies 3A10, 3F5, and 4G3, which recognize epitopes near the receptor binding region [residues 3441–3569, 2658–2816, and 2980–3080, respectively (25)] showed essentially superimposable immunoreactivity titration curves for all three LDL samples (data not shown).

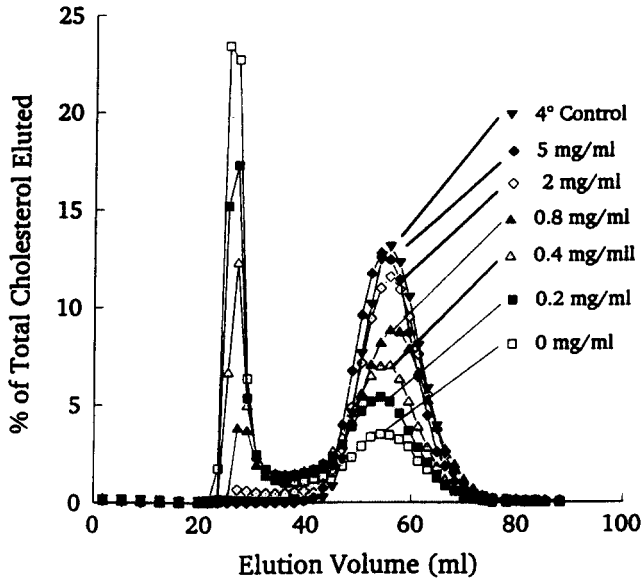


Fig. 4. Effect of sucrose concentration on freeze-thaw-induced aggregation of LDL. Sucrose was added in varying final concentrations (0–5 mg/ml) to LDL (2 mg/ml) prior to freeze-thawing. Sepharose CL2B elution profiles: samples were adjusted to the same protein concentration with saline prior to loading on the column.

In addition, there was no production of proteolytic degradation products of apoB in LDL frozen either short-term or long-term (>18 months) in 10% sucrose, as assessed by SDS-PAGE in either 4% homogeneous or 2.5–16% gradient acrylamide gels (data not shown).

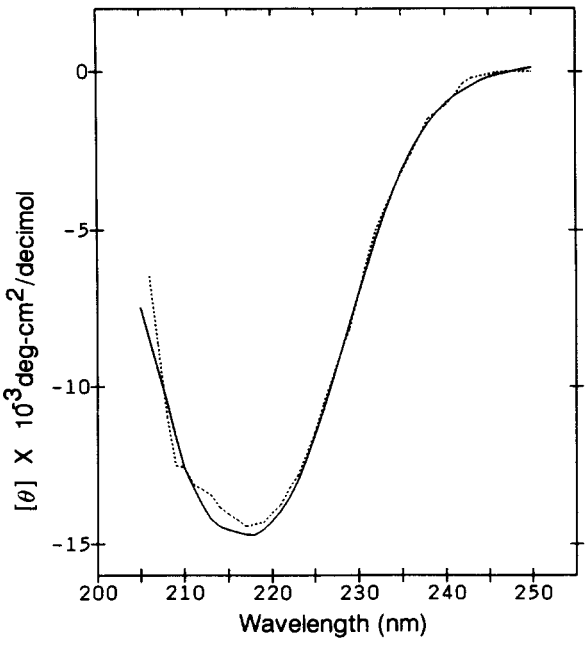


Fig. 5. Far UV circular dichroism of LDL either maintained at 4°C (—) or frozen to −70°C with 10% sucrose (w/v) (---). Ten spectra for each sample were recorded, averaged, and corrected for baseline contribution due to buffer. Sucrose frozen LDL was dialyzed against saline (150 mM NaCl, 0.24 mM EDTA, pH 7.4) prior to analysis.

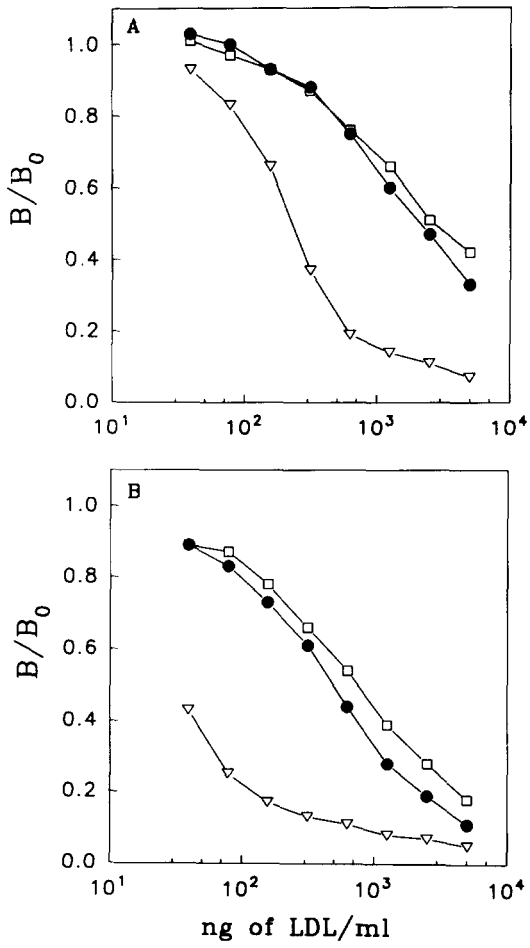


Fig. 6. Competitive radioimmunoassays of LDL with two monoclonal antibodies, 1D1 (A) and 2D8 (B), that recognize epitopes of apoB distant from the LDL binding region (see Methods). LDL samples were maintained at 4°C (●), or frozen to −70°C for 20 h with (□) or without (▽) 10% sucrose (w/v).

Cell studies

We questioned whether LDL cryopreserved in sucrose maintained normal biological properties. LDL samples that were frozen either short-term or long-term, with or without sucrose (10% w/v), were assayed for their ability to bind to cultured human fibroblasts at 4°C as well as for their binding, uptake, and degradation at 37°C. Because LDL when stored at 4°C for long periods of time can undergo both oxidative and proteolytic alterations (1–5), LDL samples cryopreserved in sucrose for long periods (up to 18 months) were compared with a freshly isolated LDL from a normolipidemic donor instead of a 4°C stored control. LDL samples that were iodinated before or after freeze–thawing displayed identical susceptibility to freeze–thaw damage and similar binding behavior (data not shown). Representative results of 4°C binding studies are shown in **Fig. 7.** LDL samples frozen without sucrose for 20 h demonstrated markedly abnormal binding to fibroblasts at 4°C. Large increases in total binding were consistently observed and were due almost entirely to increases in nonspecific binding that was 3- to 10-fold greater than that of control LDL. Specific binding was less extensively modified by freezing, but it is likely that the very high nonspecific values obtained at high ^{125}I-labeled LDL concentrations did not allow for accurate calculations of specific binding. Such increases in nonspecific binding were prevented when LDL was frozen with 10% sucrose (w/v). Binding affinities (1.67 ± 0.5 μg/ml, 2.16 ± 0.56 μg/ml) and maximal binding capacities (135.9 ± 12.6, 147.0 ± 13.4 ng/mg cell protein) of LDL samples maintained at 4°C and LDL samples

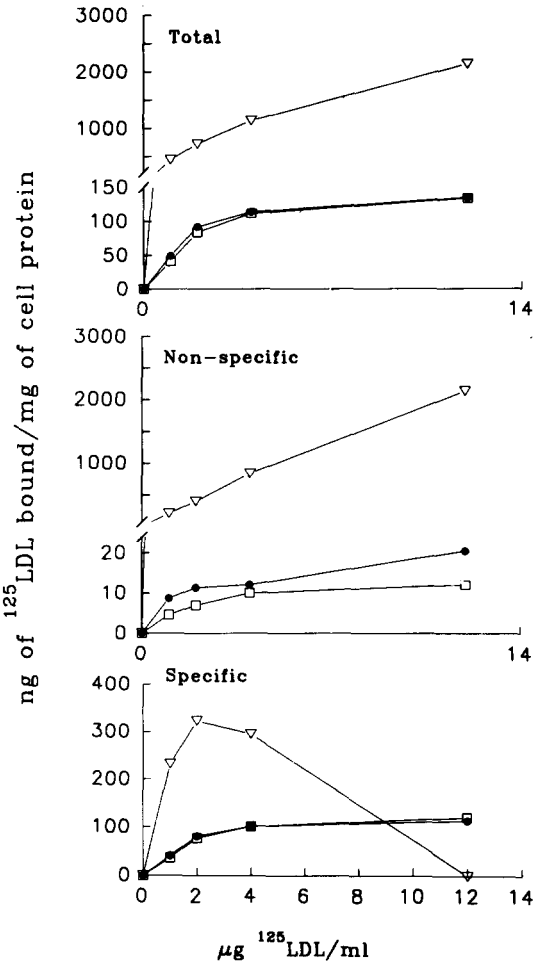


Fig. 7. Binding of LDL to fibroblast monolayers at 4°C. ^{125}I-labeled LDL (2.5 mg/ml, sp act 240 cpm/ng) was separated into three aliquots which were either stored at 4°C (●), frozen to −70°C (▽), or frozen to −70°C in the presence of sucrose 10% (w/v) (□). The frozen samples were thawed 20 h later in a 37°C water bath for 3 min and all samples were dialyzed against saline (150 mM NaCl, 0.24 mM EDTA). ^{125}I-labeled LDL fractions were added to cultured human fibroblasts in the absence (for total binding) and presence (for nonspecific binding) of 30-fold excess non-radiolabeled LDL. The cells were incubated at 4°C for 2.5 h, washed, and cell-associated radioactivity was determined. Specific binding is calculated as the difference between total and nonspecific binding. Data are the result of duplicate determinations.

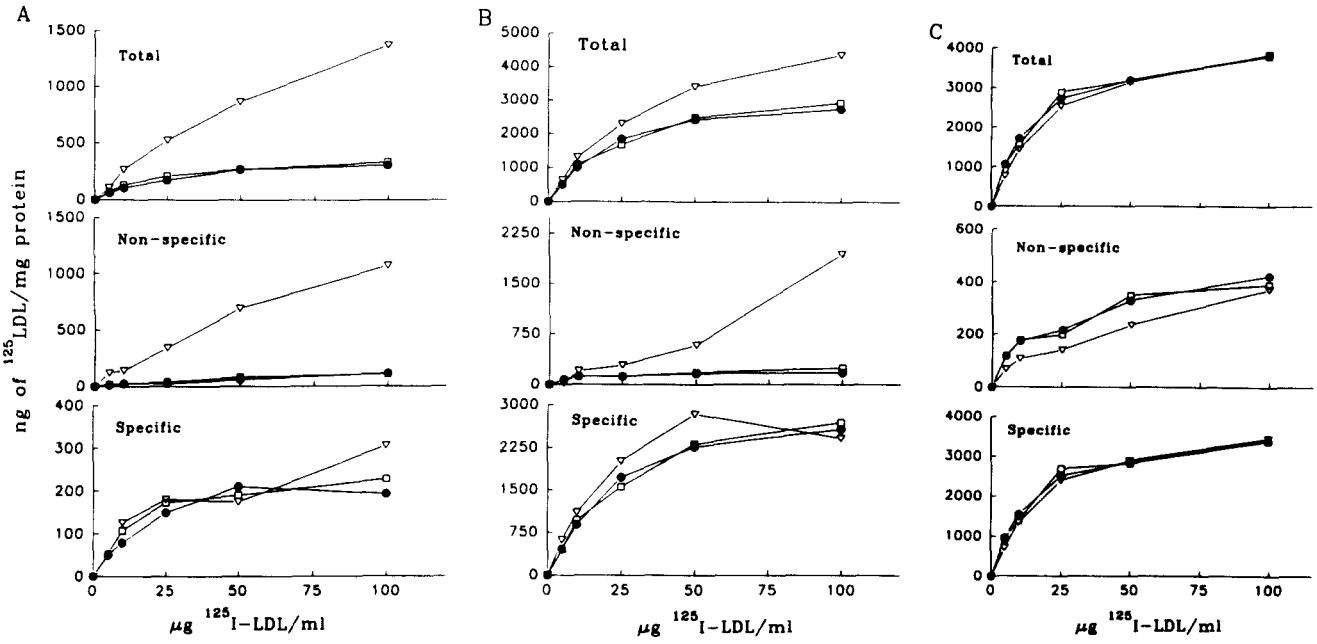


Fig. 8. Human fibroblast binding, uptake, and degradation at 37°C of ^{125}I-labeled LDL frozen for 20 h with or without sucrose. ^{125}I-labeled LDL (3 mg/ml, 255 cpm/ng) was separated into three aliquots which were stored at 4°C (●), frozen to −70°C (▽), or frozen to −70°C in the presence of 10% Sucrose (w/v) (□). After 20 h the frozen samples were thawed in a 37°C water bath and dialyzed against saline (150 mM NaCl, 0.24 mM EDTA). The three preparations were incubated with human fibroblasts for 5.5 h at 37°C. A) LDL binding assayed from radioactivity released from washed cells by a 1-h incubation with dextran sulfate buffer at 4°C; B) LDL uptake assayed from radioactivity remaining associated with the cells after dextran sulfate incubation; and C) LDL degradation measured as the non-TCA-precipitable, non iodine radioactivity in the media. Data are the means of duplicate determinations.

frozen with sucrose, respectively, were not significantly different.

Binding studies done at 37°C also demonstrated increases in total and nonspecific binding of LDL frozen without sucrose (**Fig. 8A**), which were paralleled by similar increases in total and nonspecific uptake (Fig. 8B). Degradation of frozen LDL was the same or only slightly decreased as compared with control LDL and LDL frozen with sucrose (Fig. 8C). Again, LDL frozen with sucrose showed binding, uptake, and degradation similar to LDL maintained at 4°C (Fig. 8 A, B, C).

In other experiments we compared LDL frozen in the presence of 10% sucrose (w/v) for 18 months with freshly isolated LDL (control LDL). Even after such prolonged storage, cryopreserved LDL displayed physical characteristics similar to control LDL as assessed by spectrophotometry and gel filtration, and showed 37°C binding, uptake, and degradation similar to control LDL (**Fig. 9**).

DISCUSSION

In keeping with previous studies (9, 11, 27), our results show that freeze-thawing of LDL results in irreversible alterations of its physical and biological properties. Our data demonstrate, however, that sucrose can be used as a cryopreservative agent to protect LDL solutions from the damage incurred by freezing, while maintaining normal physical, chemical, and metabolic properties after freezing for periods as long as 18 months.

Alterations in the physical characteristics of LDL due to short-term freezing were assessed utilizing several methodologies. Spectrophotometric analysis, column gel filtration, differential scanning calorimetry, and electron microscopy were utilized to assess the physical state of LDL particles. All four methods confirmed that LDL frozen with 10% sucrose (w/v) remained in solution as physically distinct particles similar to LDL maintained at 4°C, whereas LDL frozen in the absence of sucrose displayed marked aggregation. Freezing of LDL also induced fusion and/or disruption of LDL particles as visualized by electron microscopy (Fig. 2B). As well, differential scanning calorimetry analysis demonstrated that LDL frozen without sucrose displayed abnormally large transitions representative of cholesteryl ester crystal melting, suggesting LDL particle disruption and fusion (28). It has been previously demonstrated that cholesteryl ester crystals cannot form in intact human LDL particles (28). Both control LDL and LDL frozen in 10% sucrose showed normal transition profiles (R. J. Deckelbaum and S. C. Rumsey, unpublished data). No alterations were found in the secondary and tertiary structure of apoB

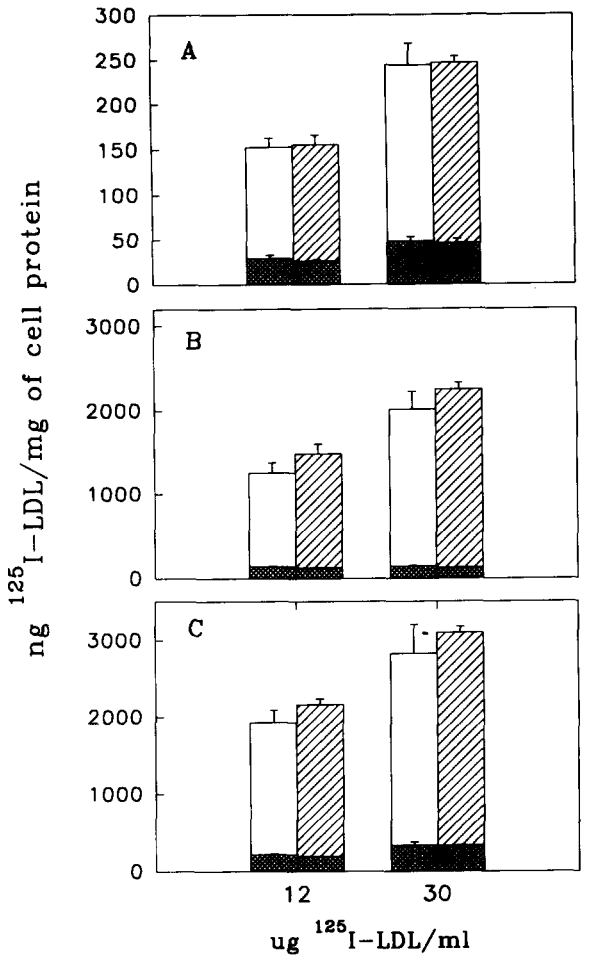


Fig. 9. Binding, uptake, and degradation at 37°C of freshly obtained LDL obtained from a normolipidemic donor and maintained at 4°C (control LDL, open bars) and LDL frozen for 18 months in 10% sucrose (hatched bars). Frozen LDL was thawed in a 37°C water bath, dialyzed against saline (150 mM NaCl, 0.24 mM EDTA) and both thawed and control LDL were iodinated (sp act: 220 and 235 cpm/ng protein, respectively). ^{125}I-labeled LDL was incubated with cultured fibroblasts at 37°C for 5.5 h at two concentrations, 12 and 30 μg/ml, and total and nonspecific binding (A), uptake (B), and degradation (C) were obtained using methods described previously. Within each bar the shaded portion represents nonspecific binding, uptake, or degradation, respectively (mean ± SD, n = 4).

after freezing with 10% sucrose using circular dichroism and monoclonal antibodies specific for various apoB epitopes. Aggregated LDL, on the other hand, displayed increased immunoreactivity to monoclonal antibodies 1D1 and 2D8, which recognize epitopes distant from the LDL receptor-binding domain (16, 25). Other antibodies (3A10, 3F5, and 4G3), which recognize epitopes associated with the LDL receptor-binding region, maintained normal binding, suggesting that the LDL receptor-binding domain is protected from the deleterious effects of freezing. It has been demonstrated that six N-linked glycosylation sites are clustered within apoB residues 3050–3450, a region corresponding to the location of the LDL receptor-binding domain (29). It is possible that glycosylation acts to sterically protect this apoB region during freezing.

We have shown that LDL frozen to −70°C displayed markedly altered binding at 4°C and binding and uptake at 37°C, and that these changes in binding behavior are primarily due to increased binding to sites other than the LDL receptor (nonspecific sites). That the sites of increased binding do not involve the LDL receptor is supported by the finding that degradation of aggregated ^{125}I-labeled LDL remains unchanged (Fig. 8C). Our data suggest that, although aggregates and fused LDL are present in large amounts in frozen LDL preparations (at least 50% of the particles), the conformation of apoB in the LDL receptor-binding domain remains largely unaffected. This is supported by the normal monoclonal antibody affinities to apoB epitopes within or near the receptor binding domain, and by 37°C cell culture studies in which the specific binding and uptake levels were comparable to control values.

Carbohydrates are common in freeze protection mechanisms in nature (30–33). In certain frogs (e.g., *R. sylvatica, H. crucifer*), exposure to very low temperatures promotes rapid tissue accumulation of glucose with subsequent protection against damage during freezing and later thawing (31, 34–36). Other organisms such as macrocysts of the slime mold Dictyostelium (32) and dry larvae of several species of nematodes (37) utilize trehalose as a protective agent against dehydration, a process that has been mechanistically compared to freezing (38). Other carbohydrates (e.g., sorbitol, ribitol, sucrose, fructose, and glycerol) have also been found in freeze-tolerant organisms (31). Studies of these natural organisms have contributed to a better understanding of mechanisms whereby carbohydrates preserve biomembranes (39–42), and serve as cyroprotectants for biological material such as oocytes, sperm, and embryos (43–45).

Studies involving freeze-drying and desiccation of phospholipid liposomes (38, 40, 41, 46–48) have demonstrated that carbohydrates prevent fusion of liposomes by forming hydrogen bonds with the phosphate head groups of the lipid particles, thereby preventing their juxtaposition. Strong evidence to support this hypothesis is provided by infrared spectral analysis of dipalmitoylphosphatidylcholine in the presence and absence of trehalose (42), and by experiments with Eu^{3+} [a molecule that binds to phospholipid head groups (49)], where with the addition of Eu^{3+} the ability of sucrose to protect liposomes against freeze damage is lost (39, 42).

LDL particles contain a neutral lipid core of cholesteryl ester and triglyceride surrounded by a monolayer of phospholipids, free cholesterol, and apoB (50). The phospholipid head groups are at the outer surface where they can presumably interact with carbohydrate in a manner similar to that which occurs with phospholipid liposomes. Although the mechanism by which LDL aggregates upon

freezing remains unclear, it seems likely that, as is the case with phospholipid liposomes, sucrose may form hydrogen bonds with phospholipid head groups and prevent aggregation-fusion of LDL by creating a barrier between LDL particles, effectively preventing contact.

The use of sucrose as a cryopreservative for LDL enables samples to be frozen to −70°C for at least 18 months without apparent alterations of structure or function. Based on these studies we cannot rule out the possibility of alterations in LDL for which we had no physical or physiological measure. In addition, whether the preservative effect of sucrose is consistent at other freezing temperatures closer to the eutectic temperature of saline (−23°C) remains an open question. As presented in this report, sucrose preservation at −70°C would be a useful way to minimize inter-experimental variability by enabling researchers to perform simultaneous comparative studies on LDL samples obtained over long intervals, and/or maintain a consistent supply of a stable "control" LDL for comparing a number of pathways involving LDL.

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