BUTTERWORTH HEINEMANN

Distribution of oxysterols in human serum: Characterization of 25-hydroxycholesterol association with serum albumin

Chen-Yi Lin* and Diane W. Morel*†

**Division of Nutrition, Department of Biochemistry, The Medical College of Pennsylvania, Philadelphia, PA USA; and †Department of Biochemistry, Medical College of Pennsylvania, Philadelphia, PA USA*

Oxysterols, derived either from cholesterol autoxidation in vitro *or through lipoprotein peroxidation* in vivo *are biologically active compounds implicated in the pathogenesis of atherosclerosis. Using 25-hydroxycholesterol (25OHC) as a model and radiolabeled 25OHC to trace mass, in this study we examined the transport of oxysterols in human serum. In contrast to cholesterol, which is associated exclusively with serum lipoproteins, 55 to 75% of 25OHC added to serum* in vitro *was associated with the lipoprotein-deficient fraction of serum (LPDS, density >1.21 g/mL) over a wide concentration range. Upon sequential gel filtration, ion-exchange chromatography, and nondenaturing polyacrylamide gel electrophoresis, 25OHC added to LPDS eluted in a single peak corresponding to a protein peak of molecular weight about 67 kD. Immunoprecipitation of serum albumin from LPDS also precipitated 25OHC. 25OHC added to albumin-depleted LPDS did not associate with any of the remaining serum proteins, suggesting that albumin is the sole protein with which 25OHC associates to any significant extent in LPDS. Bovine serum albumin (BSA) was used to characterize the association of 25OHC with albumin. Other oxysterols, including 19-hydroxycholesterol, 7β-hydroxycholesterol, and 7-keto-cholesterol, effectively competed with 25OHC for association with BSA, suggesting that they also associate with albumin in serum. The association of 25OHC with albumin in serum is selective but of low affinity as calculated from Scatchard analysis. However, given the high concentration of albumin in serum a significant amount of serum oxysterols may be delivered to or removed from various tissues via albumin.* (J. Nutr. Biochem. 6:618–625, 1995.)

Keywords: oxysterol; cholesterol; serum; lipoproteins; albumin

Introduction

Cholesterol undergoes autoxidation in air,[1,2] forming oxidized derivatives known as oxysterols. Various oxysterols, including 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, cholestane-3β, 5α, 6β-triol, epoxycholesterol, 26-hydroxycholesterol, and 25-hdyroxycholesterol (25OHC), have been detected in USP-grade cholesterol preparations[3,4] and cholesterol-containing foods,[5,7] especially in egg yolk and dairy products.[5,7] The content of oxysterols in these products has been reported to be 3 to 6 μg of dry food and to increase after processing, particularly after deep frying.[5,7] Oxysterols may enter the circulation as contaminants of cholesterol-containing food.[7] These oxysterols found in food have also been detected in human and animal plasma at concentrations of 20 to 1,200 nM (about 8 to 480 ng/mL),[8–12] and their concentrations reportedly were correlated with elevated plasma cholesterol concentrations.[12] In addition, some oxysterols may be generated by peroxidation of lipoproteins.[13,14] Administration of probucol, an antioxidant and hypocholesterolemic agent, to hypercholesterolemic rabbits decreased their plasma oxysterol concentrations, independent of its lipid-lowering effect.[15,16]

The work was supported by NIH Grant HL 45306 and the Howard Heinz Endowment.
Address reprint requests to Dr. Diane W. Morel at the Department of Biochemistry, Medical College of Pennsylvania, 2900 Queen Lane, Philadelphia, PA 19129, USA.
Received December 21, 1994; accepted May 24, 1995.

Nutritional Biochemistry 6:618–625, 1995

655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00122-G

Many oxysterols exhibit atherogenic properties and an ability to modulate cholesterol metabolism. The atherogenicity of oxysterols has been suggested by animal feeding studies in which oxysterols, when fed at relatively high doses, were more potent than pure cholesterol in causing aortic endothelial damage and inducing atherosclerosis.[3,17,18] One study showed that even at a low concentration the oxysterol cholestane-3β, 5α, 6β-triol enhanced cholesterol-induced atherosclerosis.[19] Modulation of cholesterol metabolism by various oxysterols, including cholestane-3β, 5α, 6β-triol, 26-hydroxycholesterol, and 25OHC, occurs via inhibition of the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase,[20,21] enhancement of cholesterol esterification,[22–24] and reduction of cellular cholesterol efflux.[25] In addition, numerous changes in cell structure and function have been reported following the incubation of cultured cells with an oxysterol such as 25OHC, including changes in membrane composition and phospholipid packing, echinosis in human erythrocytes,[26] increased cell fragility,[27] and enhanced membrane permeability to extracellular Ca^{2+}.[28,29]

Relatively little is known about the transport and metabolism of oxysterols in serum. In the early 1980s, three research groups examined the distribution of various oxysterols in primate serum. Among these, Peng et al.[30] reported that in squirrel monkeys ingested 25OHC associated with serum lipoproteins, predominantly with very low density lipoprotein (VLDL) and low density lipoprotein (LDL). Javitt et al.[31] reported that the small amount of 26-hydroxycholesterol detected in human serum was associated mainly with LDL and high density lipoprotein (HDL). In contrast, Streuli et al.[32] reported that about 11 to 50% of various oxysterols, including 7α-hydroxycholesterol, 20α-hydroxycholesterol, and 25OHC added to human serum in vitro, were associated with the nonlipoprotein fraction of serum (density > 1.21 g/mL). In our own preliminary studies, ingested 25OHC in mildly hypercholesterolemic rabbit serum was distributed between the lipoproteins and the lipoprotein-deficient serum (Morel, unpublished data) (LPDS, $d > 1.21$ g/mL). Using 25OHC as a model, in the current studies we investigated the distribution of oxysterols in human serum in more detail, focusing on the identification and characterization of the protein(s) in LPDS, with which oxysterols associate.

Methods and Materials

Materials

The following compounds were purchased from Sigma (St. Louis, MO USA): cholesterol, 25-hydroxycholesterol, 19-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, bovine serum albumin (BSA, essentially fatty acid-free), bilirubin, cholic acid, deoxycholic acid, oleic acid, palmitic acid, and Norit A activated charcoal. 25-[26, 27-^{3}H]-hydroxycholesterol (77 Ci/mmol) and [4-^{14}C]-cholesterol (53.0 mCi/mmol) were obtained from NEN-DuPont (Wilmington, DE USA). Sephadex G-200, DEAE-Sepharose Fast Flow, Blue Sepharose 6 Fast Flow, and Dextran T-500 were from Pharmacia-LKB (Uppsala, Sweden). Goat anti-human albumin IgG and goat anti-human immunoglobulin IgG were purchased from Cappel Research Co. (Durham, NC USA).

Labeling serum in vitro

Fresh human sera were obtained from healthy volunteers between the ages of 24 and 36 years. [^{3}H]25OHC (20 μCi/mL) was first mixed with unlabeled 25OHC (0.7 mg/mL) in ethanol to achieve a specific activity of 10 μCi/mg, and the solution was evaporated under nitrogen (99.9% pure). The dried [^{3}H]25OHC was then redissolved in 10 to 20 μL of ethanol and dispersed into 3 mL of human sera in vitro to achieve final concentrations of 0 to 100 μM (ethanol < 1%). In some experiments, serum was also labeled with [^{14}C]cholesterol (20 μCi/mL) in a similar fashion but with no added unlabeled cholesterol. Following incubation at room temperature for 1 hr then at 4°C for 18 hr, solid potassium bromide (KBr) was added to the labeled serum to adjust the density of the serum to $d = 1.210$ g/mL. The labeled serum samples were subjected to ultracentrifugation at 50,000 rpm (4°C) in a Beckman 50Ti rotor for 48 hr to isolate a single fraction of total lipoproteins and LPDS.[33] All LPDS preparations were isolated under the same conditions.

Purification of 25OHC carrier-protein in humans LPDS

LPDS, isolated from unlabeled human serum and then dialyzed in phosphate buffer saline (PBS) containing 2 mM ethylene-bis (oxyethylenenitriol) tetraacetic acid (EDTA), was labeled with [^{3}H]25OHC (20 μCi/mL) as described above except that, in order to increase the [^{3}H]25OHC specific radioactivity in the labeled LPDS, no unlabeled 25OHC mass was added. After incubation at room temperature for 1 hr, then at 4°C for 18 hr, the labeled LPDS was gel filtered (Sephadex G-200, 800 × 30 mm, eluted with PBS containing 2 mM EDTA and 0.05% sodium azide, 1 mL/hr). Blue Dextran-2000 (2,000 kD), BSA (67 kD), cytochrome c (12.5 kD), and NaCl (57.5 D) were used to calibrate the column. Elution of Blue Dextran-2000, the protein standards, and LPDS protein were monitored by the absorbance at 280 nm, NaCl was monitored by conductivity, and ^{3}H radiolabeled was quantitated by liquid scintillation counting. Fractions (2.7 mL) containing radioactivity were pooled and dialyzed in 0.025 M Tris-HCl buffer, pH 8.0, then applied (about 40 mL) to a DEAE-Sepharose ion exchange column (500 × 25 mm) equilibrated with a 0.025 M Tris-HCl buffer. Elution was carried out with a linear-gradient of NaCl, from 0.05 to 0.14 M in 0.025 M Tris-HCl buffer. Eluate fractions (3.0 mL) in which radioactivity was detected were then pooled and further purified by nondenaturing polyacrylamide gel electrophoresis (PAGE). All procedures were performed at 4°C.

Nondenaturing polyacrylamide gel electrophoresis was conducted;[34] one set of the gels was stained with 0.025% Coomassie Blue R-250 while another set in duplicate was sliced with a stainless steel gel slicer into small equal pieces (about 1 mm thick). Each gel slice was then immersed in 0.5 mL of 10% sodium dodecyl sulfate (SDS) solution at room temperature for 72 hr to allow diffusion of the trapped proteins into the SDS solution. The solution was then assessed for ^{3}H radioactivity by liquid scintillation counting, protein content using the method described by Lowry et al.,[35] and protein purity by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).[36]

Preparation of albumin-deficient human LPDS

Human LPDS, after dialysis in 0.025 M Tris-HCl buffer, was depleted of albumin by elution through a Blue Sepharose 6 Fast Flow affinity column (100 × 30 mm, in 0.025 M Tris-HCl buffer with 2 mM EDTA and 0.05% sodium azide, 15 mL/hr). As described by Travis et al.,[37] this method has been shown to quantitatively and selectively remove albumin from serum without altering other serum proteins. Aliquots of the LPDS sample before

and after chromatography were assessed by SDS–PAGE to demonstrate depletion of serum albumin. No changes in the quantities of other serum proteins were detectable. The eluate from the affinity column was collected, lyophilized, and stored at −20°C. Albumin-deficient LPDS was reconstituted with PBS and labeled in vitro with [^{3}H]25OHC prior to use.

Immunoprecipitation of serum albumin

Immunoprecipitation was performed according to Marsh et al.[38] In brief, 10 μL of the humans LPDS prelabeled with [^{3}H]25OHC was mixed with increasing volumes (0 to 1,200 μL) of goat anti-human albumin IgG (21.3 mg of protein/mL). The mixture (total volume 1.2 mL) was incubated at room temperature for 1 hr with shaking at 10 min intervals and at 4°C for 18 hr. The precipitate was pelleted by centrifugation (1,000*g* for 20 min); the pellets were washed twice with cold PBS containing 0.1% SDS and 0.15% dithiothreitol (DTT) and once with cold PBS only. The lipids were extracted twice from the pellets with 2 mL of ethanol: acetone (1:1 vol/vol) and aliquots were taken for liquid scintillation counting. The remaining protein precipitate was dried under N_2, dissolved in 0.5 mL of 0.1 N NaOH, and quantitated according to Lowry et al.[35] As a negative control, goat anti-human immunoglobulin IgG (20.7 mg of protein/mL) was used instead of anti-human albumin IgG. In a parallel study, increasing amounts of [^{3}H]25OHC-labeled LPDS were added to a constant volume of anti-albumin IgG, and the experiment was carried out in a similar fashion.

Assay for [^{3}H]25-hydroxycholesterol binding

Binding of [^{3}H]25OHC to albumin was assessed using a modification of the density-gradient method of Haddad et al.[39] [^{3}H]25OHC in 20 μL of ethanol was added to 2.0 mL of BSA (essentially fatty acid free, 30 nM) to reach final concentrations of 0 to 300 nM (0 to 10 molar excess) in the presence or absence of excess (40 fold) unlabeled 25OHC. The mixture was incubated at 37°C for 18 hr before the density of the sample was adjusted to 1.21 g/mL by adding solid KBr. After ultracentrifugation (50,000 rpm for 48 hr at 5°C), the samples were collected along the gradient and analyzed for radioactivity and protein content. Virtually all unbound 25OHC was found in a thin layer (3 to 4 mm) floating on the top of the solution, whereas BSA-bound 25OHC precipitated after centrifugation. The recovery of total [^{3}H]25OHC radiolabeled was 70 to 88% in the absence of a 40 fold excess of unlabeled 25OHC and 89 to 100% in the presence of excess. To compensate for low counts in the bottom (bound) fraction, bound [^{3}H]25OHC was calculated by subtracting free [^{3}H]25OHC in the top fraction from total [^{3}H]25OHC added to the samples. Specific binding was calculated by subtracting the values for [^{3}H]25OHC binding in the presence of unlabeled 25OHC from those in the absence of unlabeled 25OHC.

Competition for association with serum albumin

BSA (essentially fatty acid free) was prelabeled with 25OHC by adding [^{3}H]25OHC (0.1 mM, 20 μCi/mmol) to 0.01 mM BSA then incubating at 4°C for 18 hr. A high (nonphysiological) concentration of [^{3}H]25OHC was used in these experiments to maximize the ratio of 25OHC to BSA (10:1) and to minimize the nonspecific association of a small amount of [^{3}H]25OHC with the wall of the tubes. Increasing concentrations of unlabeled putative BSA ligands (0 to 1.0 mM) were added to aliquots of prelabeled albumin. For bilirubin and palmitic acid, ethanolic solutions were dried under N_2 then dissolved in 20 μL of 0.1 N NaOH and added to the labeled BSA solution. Unlabeled oxysterols were dissolved in 20 μL of ethanol and added to prelabeled albumin containing 1% cholate. Deoxycholate and cholate were dissolved in 20 μL of PBS and added directly to prelabeled albumin. All these compounds were soluble at the concentrations used. The total volume of the mixture was restricted to 1.2 mL, and all mixtures were prepared in duplicate. After incubation at 20°C for 16 hr, unbound [^{3}H]25OHC was adsorbed by dextran-coated charcoal (100 μL) according to Taylor et al.,[40] and pelleted by centrifugation at 1,000*g* for 20 min. The [^{3}H]25OHC remaining in the supernatant (BSA-bound) was quantitated by liquid scintillation counting.

Results

Distribution of 25OHC in serum

Figure 1 shows that [^{3}H]25OHC radiolabel added in vitro to human serum was found associated not only with serum lipoproteins but also with LPDS ($d > 1.21$ g/mL) over a wide range of 25OHC concentrations. Within the concentration range (10 to 1,000 nM) that oxysterols including 25OHC have been detected in vivo,[8–10] about 55 to 75% of [^{3}H]25OHC radiolabel was found in the LPDS fraction. A similar distribution was seen even at a high nonphysiological concentration of 100 μM (data not shown). In contrast, when [^{14}C]cholesterol radiolabel was added to serum to trace the distribution of endogenous serum cholesterol, virtually no [^{14}C]cholesterol radiolabel was found in the LPDS fraction. Thus, unlike cholesterol that associated exclusively with the serum lipoproteins, 25OHC associated with both lipoproteins and LPDS.

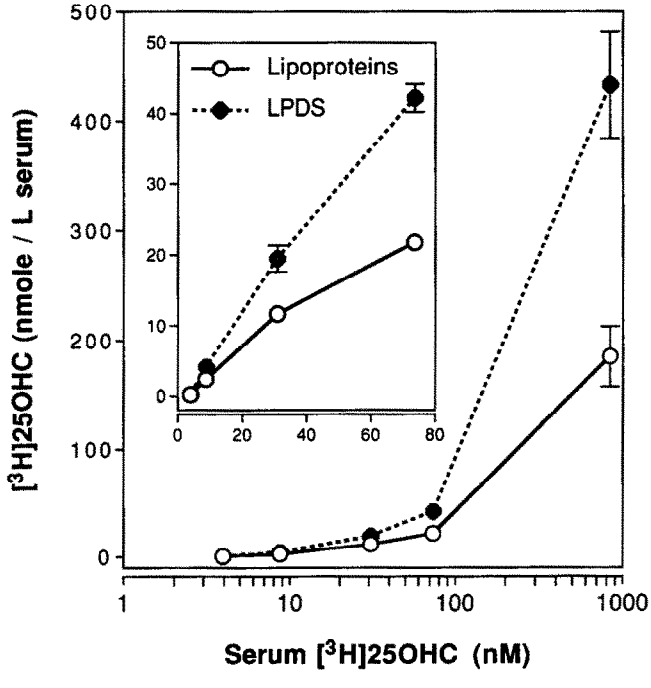

Figure 1 Distribution of [^{3}H]25OHC between lipoproteins and lipoprotein-deficient serum (LPDS) in human serum. As described in the Materials and Methods, increasing amounts of [^{3}H]25OHC (10 μCi/mg) were added to 3 mL of human serum in vitro, incubated, and then fractionated into a lipoprotein-containing fraction ($d < 1.21$ g/mL) and a LPDS fraction ($d > 1.21$ g/mL) by density ultracentrifugation. Data shown are mean ± SD for triplicate samples.

Purification of protein with which 25OHC associates in human LPDS

To determine whether oxysterols in LPDS were free or protein-associated in solution, [^{3}H]25OHC-labeled human LPDS was subjected to gel filtration on a Sephadex G-200 column (*Figure 2A*). The [^{3}H]25OHC radiolabeled eluted in a single peak corresponding to a protein peak with a molecular weight of about 60 to 70 kD, which is close to that of a BSA standard. The recovery of [^{3}H]25OHC radiolabel in this protein peak was 81%, suggesting that most of [^{3}H]25OHC in LPDS was protein-associated. To further characterize the protein(s) with which 25OHC associated, the fractions containing [^{3}H]25OHC radioactivity were pooled, dialyzed, and then eluted through an ion exchange column (DEAE-Sepharose) under a linear gradient of NaCl. Again, a single peak of [^{3}H]25OHC radiolabel was observed (*Figure 2B*). The purity of the samples after each of these steps was examined by SDS–PAGE as shown in *Figure 2C*. The predominant constituent of the fractions with which 25OHC associated was a protein with molecular weight of approximately 67 kD.

The fractions with which 25OHC associated were further analyzed by nondenaturing PAGE. As illustrated in *Figure 3*, [^{3}H]25OHC radiolabel was detected in three slices along the gel (slices 9 to 11). In a parallel set of gels, BSA migrated to a similar point in the gel (data not shown). Upon analysis by SDS–PAGE, each slice contained a single protein band with molecular weight of approximately 67 kD.

The comigration of an approximately 67 kD protein with 25OHC through gel filtration and ion exchange chromatography as well as in an electric field suggested that this protein might be a carrier protein for 25OHC in serum. The similarity in its apparent molecular weight with that of serum albumin (67 kD) suggested that this protein might be albumin.

Immunoprecipitation of protein-associated 25OHC

To ask directly if albumin is a carrier protein for 25OHC in human LPDS, a goat-derived anti-human albumin IgG was

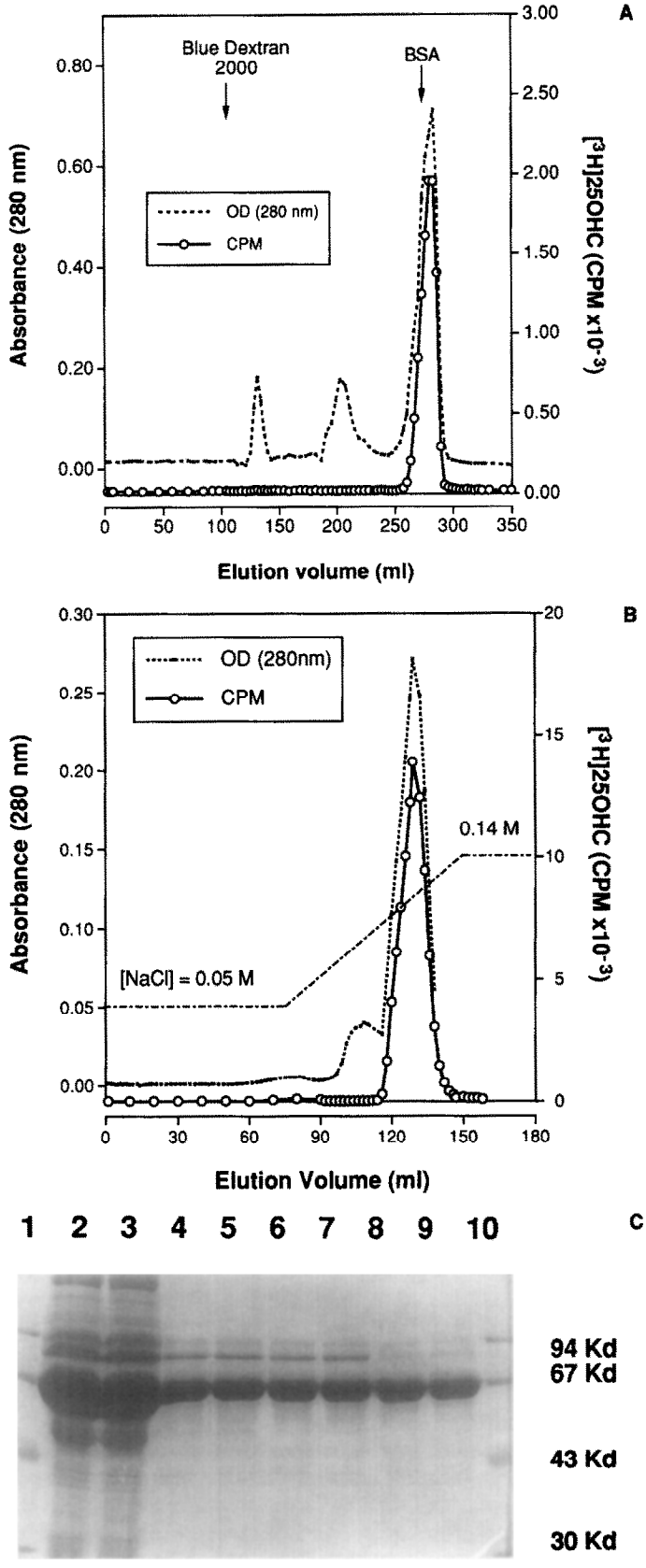

Figure 2 (A) Gel filtration of human LPDS labeled in vitro with [^{3}H]25OHC (Sephadex G-200, 800 × 30 mm). The calibrated elution volumes for Blue Dextran 2000 (2,000 kD) and BSA (67 kD) are shown by arrows. Elution of protein was monitored by absorbance at 280 nm and [^{3}H]25OHC radioactivity by liquid scintillation counting. The recoveries of ^{3}H radioactivity and protein were 81% and 82%, respectively. The [^{3}H]25OHC peak-fractions (elution volume 260 to 290 mL) from gel filtration chromatography were collected, pooled, and dialyzed in 0.025 M Tris-HCl (pH = 8.0) buffer. (B) Dialyzed sample (20 mL) was then applied to a DEAE-Sepharose ion exchange column (500 × 25 mm) pre-equilibrated with the same buffer. Elution was carried out with a linear-gradient of NaCl, from 0.05 to 0.14 M, in the above buffer. The recoveries of ^{3}H radioactivity and protein were 82% and 79%, respectively. (C) SDS–PAGE of human serum samples during steps of 25OHC-carrier protein purification. Lanes 1 and 10: molecular weight standards; lanes 2 and 3: duplicate samples of human LPDS; lanes 4 and 5: [^{3}H]25OHC peak fractions obtained after gel filtration; lanes 6 and 7: dialyzed gel filtration samples; and lanes 8 and 9: [^{3}H]25OHC peak fractions after ion exchange chromatography.

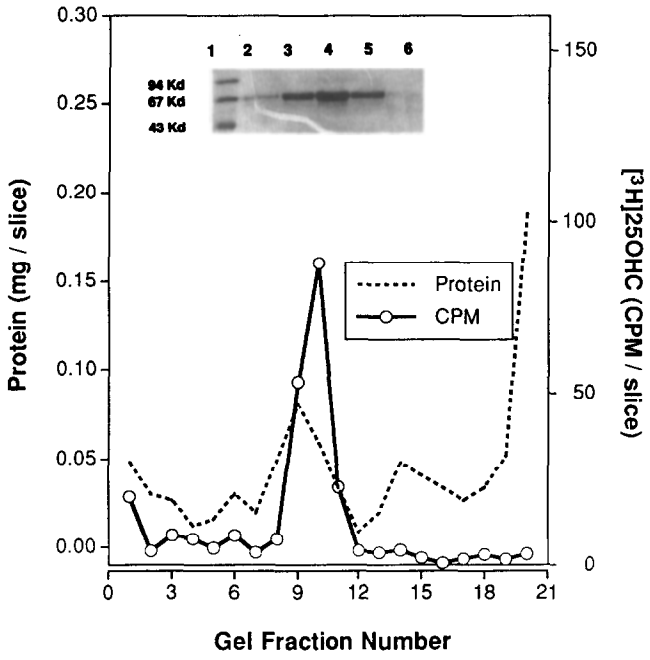

Figure 3 Nondenaturing PAGE of protein(s) with which 25OHC associates. Fractions 120 to 145 collected after ion exchange (DEAE-Sepharose) were pooled and subjected to electrophoresis in a 10% polyacrylamide tube gel. The gels were sliced and immersed in 10% SDS solution to let diffusion of trapped proteins and [^{3}H]25OHC to the solution. Aliquots were assayed by liquid scintillation counting and absorbance at 280 nm. Purity of protein was checked by SDS–PAGE. Values for protein and radioactivity are means from duplicate samples. The recoveries for ^{3}H radioactivity and protein were 84% and 79%, respectively. Insert: SDS–PAGE. Lane 1: molecular weight standards; lanes 2 to 6: samples from gel fractions 8 to 12.

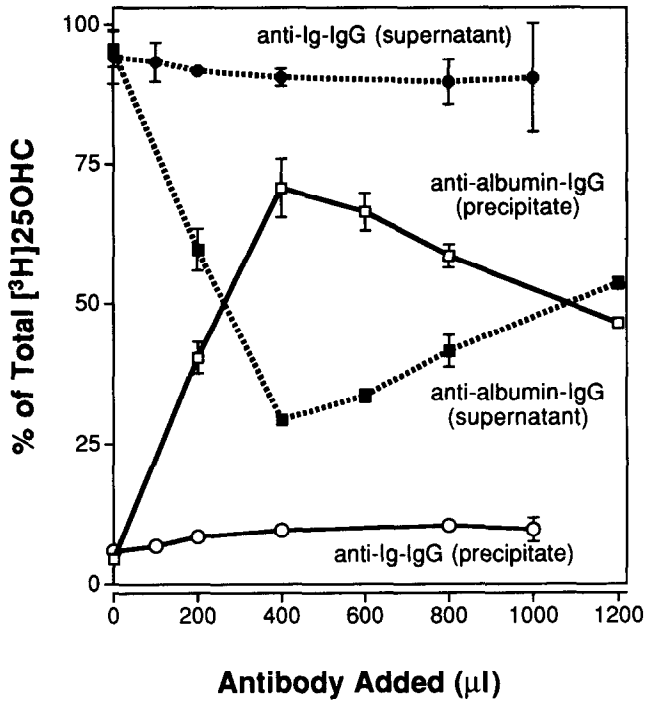

Figure 4 Immunoprecipitation of serum albumin from human LPDS labeled with [^{3}H]25OHC. Ten microliters of [^{3}H]25OHC-labeled human LPDS (45 mg of protein/mL) was mixed with increasing volumes of goat-derived IgG against either human-albumin or human-immunoglobulins (21.3 and 20.7 mg of protein/mL) as indicated. Protein precipitate (immune complex) was pelleted by centrifugation, and both the pellet and the supernatant were assayed for [^{3}H]25OHC radioactivities. Data represent the percent of total [^{3}H]25OHC in each fraction and are means ± SD for triplicate samples.

used to immunoprecipitate serum albumin from the LPDS labeled with [^{3}H]25OHC. As illustrated in *Figure 4*, this antibody, but not an anti-immunoglobulin IgG, precipitated [^{3}H]25OHC from LPDS in an antibody dose-dependent manner. In this experiment, at a maximum, about 73% of the total ^{3}H radiolabel in the mixture was precipitated by anti-albumin IgG, and virtually no albumin remained in the supernatant (SDS–PAGE; gel not shown). In another experiment, increasing amounts of the antigen ([^{3}H]25OHC-labeled LPDS) added to a constant mass of the antibody (anti-albumin IgG) precipitated at maximum more than 95% of [^{3}H]25OHC radiolabel (data not shown). These data confirm that the protein with which 25OHC associates in LPDS is serum albumin.

Association of 25OHC with albumin-depleted LPDS

To determine whether the high concentration of albumin in serum might mask a potential association between 25OHC and any other serum proteins, human LPDS was first depleted of albumin by elution through a Blue Sepharose affinity column then labeled with [^{3}H]25OHC in vitro and gel filtered (Sephadex G-200). As shown in *Figure 5A* (insert), the Blue Sepharose–treated LPDS was markedly depleted of albumin. Upon gel filtration of the labeled albumin-depleted LPDS, only 6% of the applied [^{3}H]25OHC radioactivity was recovered in the eluate, suggesting that even in the absence of albumin, 25OHC did not associate to any significant extent with other serum proteins.

To determine whether serum albumin could selectively remove [^{3}H]25OHC retained in the column, the column was washed with a mixture of proteins of different molecular weights containing BSA (67 kD) and ovalbumin (43 kD). As shown in *Figure 5B*, the [^{3}H]25OHC retained in the column was completely eluted in two peaks; about 73% of the total ^{3}H radiolabel eluted in a peak corresponding to a protein peak of molecular weight approximately 120 to 130 kD, while the remaining 27% of ^{3}H radiolabel eluted with a protein peak of approximately 60 to 70 kD. No ^{3}H radiolabel was associated with the 40 to 50 kD peak of ovalbumin. Upon SDS–PAGE (*Figure 5C*), both protein peaks with which [^{3}H]25OHC associated contained a predominant band with a molecular weight identical to that of BSA. Thus, the higher molecular weight (120 to 130 kD) protein with which [^{3}H]25OHC associated was a dimer of BSA.

Characterization of the association between 25OHC and albumin

A number of oxysterols, including 19-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol, were tested for their abilities to compete with [^{3}H]25OHC for association with BSA. There results are shown in *Figure 6A*. Each

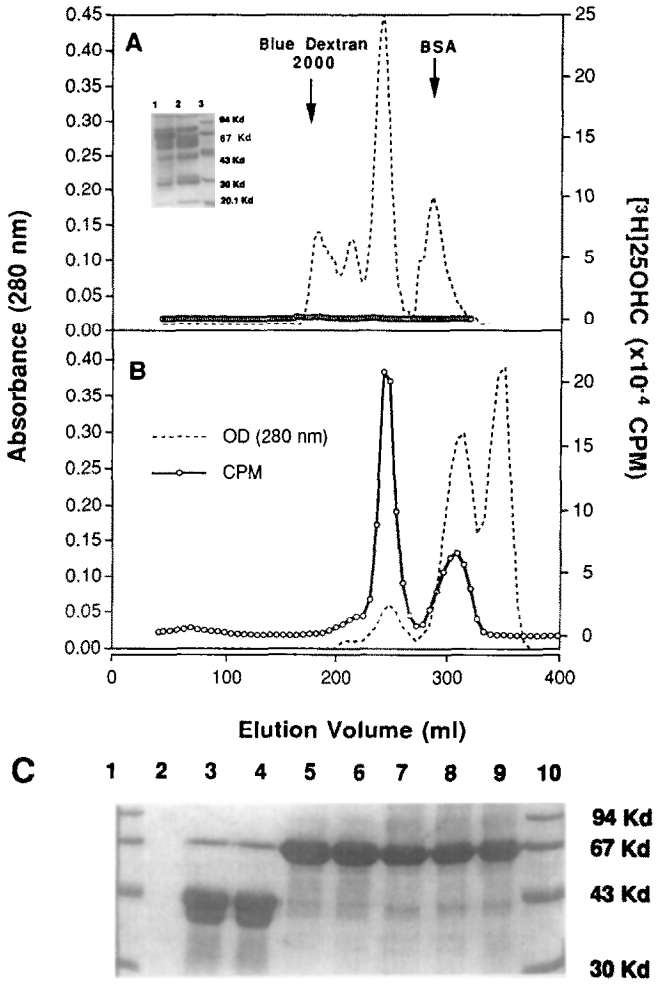

Figure 5 Gel filtration of albumin-deficient human LPDS after labeling with [^{3}H]25OHC (50 μCi/mL). (A) Elution with PBS containing 2 mM EDTA and 0.01% sodium azide. Insert: SDS–PAGE of human LPDS before (lane 1) and after (lane 2) blue Sepharose affinity chromatography. Lane 3: molecular weight standards. (B) Elution with 10 mL of 2% BSA and 2% ovalbumin in PBS. Protein content was monitored by absorbance at 280 nm and [^{3}H]25OHC radioactivity by liquid scintillation counting. (C) SDS–PAGE of protein-peak samples from the BSA/ovalbumin elution: molecular weight standards in lanes 1 and 10; pooled fractions from the BSA/ovalbumin elution in lanes 3 and 4 (325 to 375 mL), lanes 5 and 6 (275 to 324 mL), and lanes 7 to 9 (220 to 274 mL).

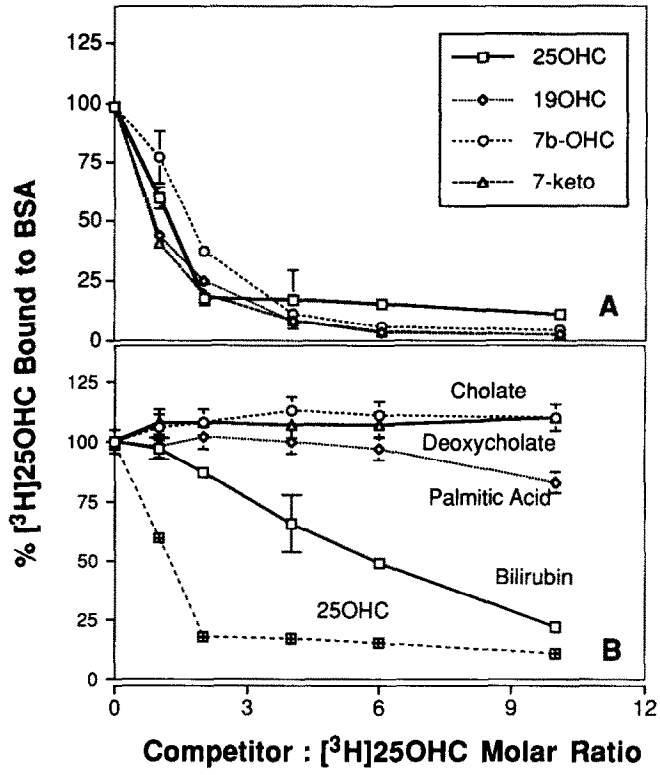

Figure 6 Competition for association with albumin by other oxysterols, albumin ligands and biological detergents. [^{3}H]25OHC (0.1 mM) in ethanol was added to BSA (essentially fatty acid free, 0.01 mM) in vitro to yield a 25OHC to BSA ratio of 10:1. Other oxysterols and known albumin ligands were then added to (as described in the Methods and materials section) 1 mL of [^{3}H]25OHC-labeled BSA to achieve the indicated competitor-to-[^{3}H]25OHC molar ratios. Charcoal-dextran was used to adsorb free [^{3}H]25OHC and competing ligands and removed by centrifugation (1,000*g*). The ^{3}H radiolabel remaining in the supernatant was BSA-associated. Data shown are means ± range for duplicate samples.

oxysterol effectively decreased the association of [^{3}H]25OHC with BSA in a dose-dependent manner; maximal competition (10% if origin) was seen at a 10 fold molar excess of unlabeled oxysterol. It is thus likely that other oxysterols exhibit a similar distribution in serum, i.e., they also associate with albumin. We also tested whether the putative ligands of albumin, including free fatty acids, bilirubin, and bile acids (cholate and deoxycholate, also biological detergents) could limit the association of [^{3}H]25OHC with BSA. As shown in *Figure 6B*, bilirubin reduced the association of [^{3}H]25OHC with albumin in a dose-dependent manner to 20% of its original value. In contrast, free fatty acids (palmitic or oleic) and bile salts (cholate or deoxycholate), up to a 10 fold molar excess, had no effect on the association of radiolabeled 25OHC with BSA. Progesterone, estradiol, cortisone, corticosterone, and hydrocortisone were also ineffective competitors (data not shown).

To further examine the nature of the interaction between 25OHC and albumin, a binding assay was performed as described in the Methods and Materials section. The association of increasing concentrations of labeled 25OHC with fatty acid-free BSA in the presence and absence of a 40 fold excess of unlabeled 25OHC was examined. Scatchard analysis[41] of these data suggested a K_d of 5×10^{-4}M and a B_{max} of 0.11 mol of 25OHC/mol of albumin, values indicative of a low affinity association with a maximum binding of one molecule of 25OHC per ten molecules of BSA. Other methods of assessing 25OHC binding, such as dextran-charcoal precipitation of unbound oxysterol as described by Dawson et al.[42] and a solid-phase binding assay,[43] yielded similar binding curves and Scatchard analysis results (data not shown).

Discussion

These studies show that 25-hydroxycholesterol, a biologically active oxysterol, distributes between lipoproteins and

albumin in serum. These findings agree with and extend the observations of Streuli et al.[32] that oxysterols associate in vitro with the "nonlipoprotein serum" fraction. The identification of albumin as the sole protein in LPDS with which 25OHC associates suggests that albumin may serve as a transport protein for oxysterols. Moreover, given the ability of other oxysterols to compete with labeled 25OHC for association with BSA, it is likely that they too associate with albumin in serum.

The distribution of 25OHC differs markedly from that of cholesterol, which is associated exclusively with lipoprotein in serum. Part of this difference may be explained by the greater polarity of 25OHC and other oxysterols. Their greater solubilities in an aqueous environment may allow them to interact with albumin more readily. Despite the apparent low affinity, the association so formed is stable to numerous manipulations including ultracentrifugation and electrophoresis.

Despite the structural similarities between oxysterols and other steroids, such as corticosteroids and vitamin D (1,25-dihydroxycholecalciferol) which bind to specific transport-proteins in serum,[44,45] our findings suggest that albumin is the sole nonlipoprotein plasma protein with which 25OHC associates to any significant extent. From this point of view, the association of 25OHC with albumin is selective and specific. It is known that serum albumin associates with many hydrophobic compounds in circulation.[46] Among these, long-chain free fatty acids and bilirubin bind to albumin with high affinity and low capacity,[46] while others such as progesterone and testosterone bind to albumin with low affinity and high capacity.[44] Given the structural similarity between steroids and oxysterols, it is not entirely surprising that the interaction between albumin and 25OHC is of low affinity. This interaction likely depends on hydrophobic attraction between the oxysterol molecule and the hydrophobic pocket (or domain) of the albumin molecule. This speculation is supported by the apparent lack of stereospecificity for oxysterol association with albumin as well as by the observation that bilirubin, a molecule distinct in structure from that of oxysterols which binds to a specific binding site on albumin,[46] also inhibited the association of oxysterol with albumin. It is likely that the interaction between oxysterol and albumin occurs near the bilirubin-binding region, or that the binding of bilirubin to albumin alters its conformation such that oxysterol can no longer associate. However, exactly how approximately one molecule of 25OHC actually interacts with ten molecules of albumin on a molecular basis is not clear. Apparently, though, this interaction between oxysterols and hydrophobic domain(s) of albumin remains stable even in the presence of biological detergents such as cholate and deoxycholate.

From our chromatography studies, 25OHC appeared to bind more readily to the dimeric form of albumin than to the monomer. In many animal species, including rat, rabbit, guinea pig, and human, preferential binding of serum sterols to multimeric forms of their binding proteins has been reported.[47,48] While the reason for this is not clear, it is possible that the number of ligand-binding sites is greater in the multimer than in the monomer, as has been reported for the binding of progesterone to BSA.[49]

Very recently, Bjorkhem et al.[50] have provided evidence showing that cholesterol loading in human alveolar macrophages effectively enhanced the conversion of cholesterol to oxysterols and the secretion of oxysterols into the medium. This secretion of cellular oxysterols required the presence of serum proteins in the culture medium. Based on these observations, the investigators predicted the presence of an oxysterol-carrier protein in serum that could facilitate the removal of cellular cholesterol in the form of oxysterols. Our findings suggest that albumin is this oxysterol-carrier protein and, in addition, other data from our laboratory[25] have demonstrated that BSA can stimulate efflux of 25OHC from a variety of cells in culture. Given the high concentration of albumin in serum, the capacity of albumin for oxysterol association is significant. It is possible that a significant amount of oxysterol converted from cholesterol is secreted and removed by albumin. Thus, it may be, as hypothesized by Bjorkhem et al.,[50] that conversion of cellular cholesterol to oxysterols represents a general defense mechanism to counteract elevated intracellular cholesterol levels. However, it is not clear whether such reverse transport of oxysterols to the liver for further catabolism occurs in vivo and functions to limit atherosclerosis, or whether the other proatherogenic effects of oxysterols, including cytotoxicity,[51–53] enhancement of cholesterol esterification,[22–24] and reduction of cholesterol efflux[25] prevail.

Acknowledgment

The authors are grateful to Drs. A. Catharine Ross and Julian B. Marsh for fruitful discussion.

References

1 Smith, L.L. (1987). Cholesterol autoxidation. *Chem. Phys. Lipids* **44,** 87–125
2 Kim, S.K. and Nawar, W.W. (1993). Parameters influencing cholesterol oxidation. *Lipids* **28,** 917–922
3 Imai, H., Werthessen, N.T., Taylor, C.B., and Lee, K.T. (1976). Angiotoxicity and atherosclerosis due to contaminants of USP-grade cholesterol. *Arch. Pathol. Lab. Med.* **100,** 565–572
4 Taylor, C.B., Peng, S.K., Werthessen, N.T., Tham, P., and Lee, K.T. (1979). Spontaneously occurring angiotoxic derivatives of cholesterol. *Am. J. Clin. Nutr.* **32,** 40–57
5. van de Bovenkamp, P., Kosmeijer-Schuil, T.G., and Katan, M.B. (1988). Quantification of oxysterols in Dutch foods: Egg products and mixed diets. *Lipids* **23,** 1079–1085
6 Addis, P.B. (1986). Occurrence of lipid oxidation products in foods. *Food Chem. Toxicol.* **24,** 1021–1030
7 Kumar, N. and Singhal, O.P. (1991). Cholesterol oxides and atherosclerosis: a review. *J. Sci. Food Agric.* **55,** 497–510
8 Breuer, O. and Bjorkhem, I. (1990). Simultaneous quantification of several cholesterol autoxidation and monohydroxylation products by isotope-dilution mass spectrometry. *Steroids* **55,** 185–192
9 Stalenhoef, A.F.H., Kleinveld, H.A., Kosmeijer-Schuil, T.G., Demacker, P.N.M., and Katan, M.B. (1993). In vivo oxidized cholesterol in atherosclerosis. *Atherosclerosis* **98,** 113–114
10 Harik-Khan, R. and Holmes, R.P. (1990). Estimation of 26-hydroxycholesterol in serum by high-performance liquid chromatography and its measurement in patients with atherosclerosis. *J. Steroid Biochem.* **36,** 351–355
11 Koopman, B.J., van der Molen, J.C., and Wolthers, B.G. (1987). Determination of some hydroxycholesterols in human serum samples. *J. Chromatog.* **416,** 1–13
12 Hodis, H.N., Crawford, D.W., and Sevanian, A. (1991). Cholesterol feeding increases plasma and aortic tissue cholesterol oxide

levels in parallel: Further evidence for the role of cholesterol oxidation in atherosclerosis. *Atherosclerosis* **89,** 117–126

13 Hodis, H.N., Kramsch, D.M., Avogaro, P., Bittolo-Bon, G., Cazzolato, G., Hwang, J., Peterson, H., and Sevanian, A. (1994). Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL). *J. Lipid Res.* **35,** 669–677

14 Hughes, H., Mathews, B., Lenz, M.L., and Guyton, J.R. (1994). Cytotoxicity of oxidized LDL to porcine smooth muscle cells is associated with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. *Arterioscler. Thromb.* **14,** 1177–1185

15 Masana, L., Bargallo, M.T., Plana, N., LaVille, A., Casals, I., and Sola, R. (1991). Effectiveness of probucol in reducing plasma low-density lipoprotein cholesterol oxidation in hypercholesterolemia. *Am. J. Cardiol.* **68,** 863–867

16 Hodis, H.N., Chauban, A., Hashimoto, S., Crawford, D.W., and Sevanian, A. (1992). Probucol reduces plasma and aortic wall oxysterol levels in cholesterol fed rabbits independently of its plasma cholesterol lowering effect. *Atherosclerosis* **96,** 125–134

17 Morin, R.J., and Peng, S.K. (1989). The role of cholesterol oxidation products in the pathogenesis of atherosclerosis. *Ann. Clin. Lab. Sci.* **19,** 225–237

18 Hubbard, R.W., Ono, Y., and Sanchez, A. (1989). Atherogenic effect of oxidized products of cholesterol. *Prog. Food Nutri. Sci.* **13,** 17–44

19 Jacobson, M.S., Price, M.G., Shamoo, A.E., and Heald, F.P. (1985). Atherosclerosis in white Carneau pigeons. Effects of low-level cholestane-triol feeding. *Atherosclerosis* **57,** 209–217

20 Kandutsch, A.A., Chen, H.W., and Heiniger, H.J. (1978). Biological activity of some oxygenated sterols. *Science* **201,** 498–501

21 Kandutsch, A.A., and Chen, H.W. (1975). Regulation of sterol synthesis in cultured cells by oxygenated derivatives of cholesterol. *J. Cell Physiol.* **85,** 415–424

22 Brown, M.S., Dana, S.E., and Goldstein, J.L. (1975). Cholesterol ester formation in cultured human fibroblasts. Stimulation by oxygenated sterols. *J. Biol. Chem.* **250,** 4025–4027

23 Kusuhara, H., Shimada, O., and Inui, J. (1992). Effect of 25-hydroxycholesterol on cholesteryl ester formation in Caco-2 cells. *Lipids* **27,** 478–480

24 Field, F.J., and Mathur, S.N. (1983). Regulation of acyl CoA: cholesterol acyltransferase by 25-hydroxycholesterol in rabbit intestinal microsomes and absorptive cells. *J. Lipid Res.* **24,** 1049–1059

25 Kilsdonk, E.P.C., Morel, D.W., Johnson, W.J., and Rothblat, G.H. (1995). Inhibition of cellular cholesterol efflux by 25-hydroxycholesterol. *J. Lipid Res.* **36,** 505–516

26 Streuli, R.A., Kanofsky, J.R., Gunn, R.B., and Yachnin, S. (1981). Diminished osmotic fragility of human erythrocytes following the membrane insertion of oxygenated sterol compounds. *Blood* **58,** 317–325

27 Theunissen, J.J., Jackson, R.L., Kempen, H.J., and Demel, R.A. (1986). Membrane properties of oxysterols. Interfacial orientation, influence on membrane permeability and redistribution between membranes. *Biochim. Biophys. Acta* **860,** 66–74

28 Boissonneault, G.A., and Heiniger, H.J. (1984). 25-Hydroxycholesterol-induced elevation in 45Ca uptake: Correlation with depressed DNA synthesis. *J. Cell Physiol.* **120,** 151–156

29 Boissonneault, G.A., and Heiniger, H.J. (1985). 25-Hydroxycholesterol-induced elevations in 45Ca uptake: permeability changes in P815 cells. *J. Cell Physiol.* **125,** 471–475

30 Peng, S.K., Taylor, C.B., Mosbach, E.H., Huang, W.Y., Hill, J., and Mikkelson, B. (1982). Distribution of 25-hydroxycholesterol in plasma lipoproteins and its role in atherogenesis. A study in squirrel monkeys. *Atherosclerosis* **41,** 395–402

31 Javitt, N.B., Kok, E., Burstein, S., Cohen, B., and Kutscher, J. (1981). 26-Hydroxycholesterol. Identification and quantitation in human serum. *J. Biol. Chem.* **256,** 12644–12646

32 Streuli, R.A., Chung, J., Scanu, A.M., and Yachnin, S. (1981). Serum lipoproteins modulate oxygenated sterol insertion into human red cell membranes. *Science* **212,** 1294–1296

33 Havel, R.J., Eder, H.A., and Bragdon, J.J. (1955). The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34,** 1345–1353

34 Chrambach, A., and Rodbard, D. (1971). Polyacrylamide gel electrophoresis. *Science* **172,** 440–451

35 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

36 Chrambach, A., Jovin, T.M., Svsdsen, P.J., and Robard, D. (1976). Analytical and preparative polyacrylamide gel electrophoresis. An objectively defined fractionation route, apparatus and procedures. In *Methods of Protein Separation* (N. Castimpoolas, ed.), p. 27–144, Plenum Publishing Corp., New York, NY USA

37 Travis, J., and Pannell, R. (1973). Selective removal of albumin from plasma by affinity chromatography. *Clin. Chim. Acta* **49,** 49–52

38 Marsh, J.B. (1959). The synthesis of plasma lipoprotein by rat liver. *J. Biol. Chem.* **234,** 3196–3200

39 Haddad, J.G., and Walgate, J. (1976). 25-Hydroxyvitamin D transport in human plasma. Isolation and partial characterization of calcidiol-binding protein. *J. Biol. Chem.* **251,** 4803–4809

40 Taylor, F.R., and Kandutsch, A.A. (1985). Use of oxygenated sterols to probe the regulation of 3-hydroxy-3-methylglutaryl-CoA reductase and sterogenesis. *Meth. Enzymol.* **110,** 9–19

41 Scatchard, G. (1949). The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51,** 660–672

42 Dawson, P.A., Van der Westhuyzen, D.R., Goldstein, J.L., and Brown, M.S. (1989). Purification of oxysterol binding protein from hamster liver cytosol. *J. Biol. Chem.* **264,** 9046–9052

43 Kounnas, M.Z., Chappell, D.A., Strickland, D.K., and Argraves, W.S. (1993). Glycoprotein 330, a member of the low density lipoprotein receptor family, binds lipoprotein lipase in vitro. *J. Biol. Chem.* **268,** 14176–14181

44 Westphal, U. (1986). Serum albumin. In *Steroid-Protein Interactions II* (F. Gross, M.M. Grumbach, A. Labhart, M.B. Lipsett, T. Mann, L.T. Samuels, J. Zander, eds.), p. 8–22, Springer-Verlag, Berlin

45 Cooke, N.E., and Haddad, J.G. (1989). Vitamin D-binding protein (Gc-globulin). *Endocrinol. Rev.* **10,** 294–307

46 Kragh-Hensen, U. (1990). Structure and ligand binding properties of human serum albumin. *Dan. Med. Bull.* **37,** 57–84

47 Friedli, H., and Kistler, P. (1970). Polymers in preparation of human serum albumin. *Vox Sang.* **18,** 542–546

48 Rosner, W. (1972). Recent studies on the binding of cortisol in serum. *J. Steroid Biochem.* **3,** 531–542

49 Westphal, U., and Harding, G.B. (1973). Steroid-protein interactions. XXVII. Progesterone binding to polymers of human serum albumin. *Biochim. Biophys. Acta* **310,** 518–527

50 Bjorkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R.J., Duan, C., and Lund, E. (1994). Atherosclerosis and sterol 27-hydroxylase: Evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc. Natl. Acad. Sci. USA* **91,** 8592–8596

51 Peng, S.K., Tham, P., Taylor, C.B., and Mikkelson, B. (1979). Cytotoxicity of oxidation derivatives of cholesterol on cultured aortic smooth muscle cells and their effect on cholesterol biosynthesis. *Am. J. Clin. Nutr.* **32,** 1033–1042

52 Guyton, J.R., Black, B.L., and Seidel, C.L. (1990). Focal toxicity of oxysterols in vascular smooth muscle cell culture. A model of the atherosclerotic core region. *Am. J. Pathol.* **137,** 425–434

53 Peng, S.K., Taylor, C.B., Hill, J.C., and Morin, R.J. (1985). Cholesterol oxidation derivatives and arterial endothelial damage. *Atherosclerosis* **54,** 121–133