Properties of an Albumin Inhibiting Lysosomal Acid Cholesteryl Ester Hydrolase in Rat Liver

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Lysosomal acid cholesteryl ester hydrolase (acid CEH, EC. 1.1.1.13) activity was inhibited by addition of an increasing amount of $d=1.21$ bottom fraction from rat serum (Lipids in press). To clarify the mechanism of this inhibition, rat native and modified albumin were added to the assay mixture and their effects on acid CEH activity were examined. The inhibitory effect on acid CEH activity was dependent on the concentration of rat albumin. Albumin of various mammalian species, including human, bovine and rabbit, also inhibited acid CEH activity. This inhibitory activity was markedly increased by heat treatment, the effect increasing in parallel with the prolongation of the treatment. Moreover, this albumin-dependent inhibition of acid CEH activity was also markedly increased by methylation of albumin. In contrast, the inhibition of acid CEH activity by modified albumins, such as acetyl albumin, succinyl albumin and glycine methyl ester albumin, was much lower than that of albumin, and no stimulatory effect of heat treatment on the albumins was observed. The heat-treated albumin-dependent inhibition of acid CEH activity was not abolished in the presence of sodium deoxycholate. The values of V_{max} obtained were similar with or without heat-treated albumin.

These results suggest that the inhibitory effects of heat-treated albumin may be due to an intrinsic and characteristic property of the lipid/water interface, and that the stimulatory effects of heat treatment on albumin-dependent inhibition may be due to heat-induced changes in the affinity and conformation of albumin.

Keywords albumin; acid cholesteryl ester hydrolase; cholesterol metabolism; cholesteryl ester

Introduction

Lysosomal acid cholesteryl ester hydrolase (acid CEH) has been purified by chromatography on phenyl Sepharose and concanavalin A (Con A) Sepharose.[1-3] Previous studies have shown that the enzyme is a hydrophobic glycoprotein associated with the lysosomal membrane,[4] and that it also possesses activity against triglycerides.[1]

A defect of acid CEH results in various inborn disorders of metabolism, which are characterized by lipid accumulation in several organs, such as Wolmans disease[5] and cholesteryl ester storage disease.[6] It has also been recognized as an important factor in the accumulation of cholesteryl esters in atherosclerotic arteries.[7-10] However, the mechanism of regulation acid CEH activity in cellular cholesteryl ester metabolism remains unclarified.

We recently reported the inhibition of acid CEH from rat liver by apo A—I and the $d=1.21$ bottom fraction,[11] indicating that apolipoproteins may play a role in the regulation of hydrolysis of cholesteryl esters in lipoproteins. In the present study, evidence was obtained indicating that acid CEH is inhibited by albumin, which is the major protein in the $d=1.21$ bottom fraction. This report describes the effects of albumin on acid CEH activity in rat liver.

Materials and Methods

Chemicals and Radiochemicals Cholesteryl[1-^{14}C]oleate (specific activity 58.6 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Bovine brain phosphatidylserine, egg phosphatidylcholine, dicetyl phosphate, methylated bovine serum albumin, bovine serum albumin, human serum albumin, rabbit serum albumin, rat serum albumin, and polylysine were purchased from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-200, Polybuffer Exchanger 94, and Polybuffer 74 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Centriflo (Type CF-25) was purchased from Amicon Co. (Danvers, MA).

Preparation of Lysosomes Young male Sprague-Dawley rats weighing 180—200 g were killed by decapitation and the livers were perfused with ice-cold 1.15% KCl solution at 4 °C. The tissues were homogenized in 8 volumes of ice-cold 0.25 M sucrose/1 mM ethylenediaminetetraacetic acid (EDTA)/0.01 M Tris–HCl buffer (pH 7.5). The homogenate was fractionated by the method of Brecher *et al.*[12] to obtain the lysosomal fraction.

Purification of Acid CEH All preparation steps were carried out at 4 °C. Acid CEH was prepared as described previously.[13]

Acid Cholesteryl Ester Hydrolase Assay Acid CEH activity was measured by the method of Brecher *et al.*[12] The substrate contained, per 2 ml, 4 μCi cholesteryl [1-^{14}C]oleate, 0.4 μmol phosphatidylcholine, 0.4 μmol phosphatidylserine, and 0.2 μmol sodium taurocholate. After lyophilization, 1 ml of 0.1 M NaCl, 0.01 M Tris–HCl (pH 7.4), and 0.02% NaN_3 was added, and the ampules were filled with nitrogen, stoppered and shaken by vortexing. Vesicles were prepared by sonication with a Branson Sonifier for 2 min at 40 °C.

Acetylation and Succinylation of Bovine Serum Albumin Acetylation of bovine serum albumin was done as described by Ansari *et al.*[14] Bovine serum albumin (20 mg) was dissolved in 20 ml of 0.09 M sodium phosphate buffer, pH 7.4 in a reaction vessel kept at 0—4 °C with constant stirring. Acetic anhydride was added to the protein solution (100 fold molar excess with respect to protein) and the pH of the reaction mixture was maintained between 7.4 and 7.6 by the addition of 1 M NaOH. The reaction was carried out for 30 min. Succinylation of bovine serum albumin was done as described by Kidwai *et al.*[15] in 0.1 M sodium phosphate buffer (pH 7.4). Solid succinic anhydride (1000 fold molar excess with respect to protein) was added to the protein solution (1 mg/ml) over a period of 1 h with gentle stirring, and the pH of the reaction mixture was maintained by addition of 0.2 M NaOH. The solution was left for 30 min at room temperature (25 °C). Both reaction mixtures were dialyzed first against distilled water and several changes of 0.15 M phosphate buffer, pH 7.0 and concentrated by membrane filtration. The extent of chemical modification of the lysine residue of bovine serum albumin was determined by the ninhydrin method of Moore and Stein.[16] The percentage of modification of acetylated and succinylated bovine serum albumin was about 80% and 85%, respectively.

Preparation of Lipoprotein Fractions Lipoproteins were separated from sera of male Sprague-Dawley rats (250 g body weight) as described by Hatch and lees.[17] The animals were fasted for 24 h before bleeding. Density criteria for the various lipoprotein fractions were as follows: $d<1.006$ for very low density lipoprotein (VLDL), $d=1.019$—1.040 for low density lipoprotein (LDL) and $d=1.063$—1.21 for high density lipoprotein (HDL). The $d=1.21$ bottom fraction was obtained from the lower half of the centrifugation tubes of the last centrifugation.

Results

Effect of the $d=1.21$ Bottom Fraction or Albumin on Acid CEH Activity The activity of acid CEH was determined in the presence of increasing concentrations of the $d=1.21$ bottom fraction or albumin. As shown in Fig. 1, the acid CEH activity was inhibited by addition of about 45 μg and 14 μg of the $d=1.21$ bottom fraction or albumin, respectively, the inhibition being approximately half-maximal.

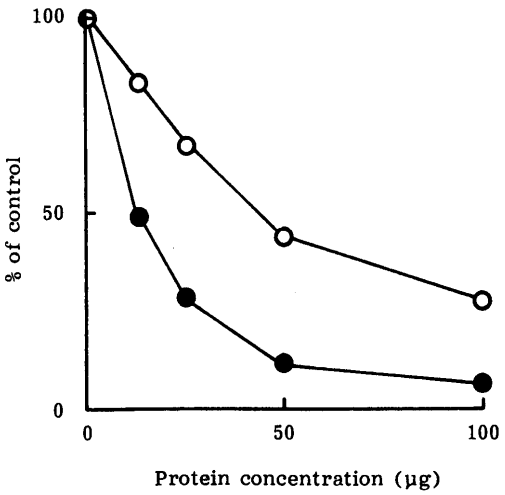

Fig. 1. Effect of the $d=1.21$ Bottom Fraction or Albumin on Acid CEH Activity

The 100% value is the activity under standard assay conditions. ○, $d=1.21$ bottom fraction; ●, rat serum albumin.

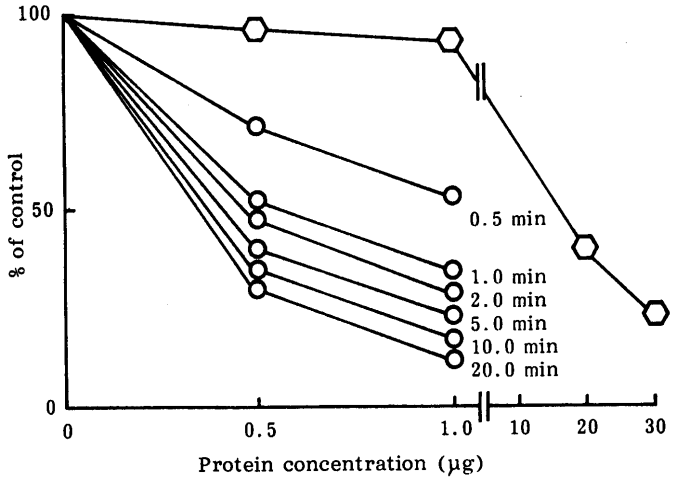

Fig. 2. Effect of Albumin Heated at 100 °C for Various Periods with pH 7.0 on Acid CEH Activity

Bovine serum albumin (100 μg/ml) was heated at 100 °C in 0.05 M Tris–HCl buffer at pH 7.0. The 100% value is the activity under standard assay conditions. ○, native bovine serum albumin; ○, heat-treated bovine serum albumin.

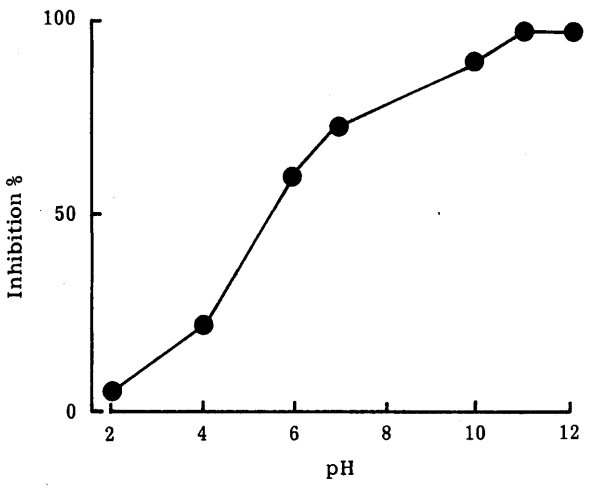

Fig. 3. Effect of Albumin Heated at Various pH Values on Acid CEH Activity

Bovine serum albumin (100 μg/ml) was heated at various pH levels for 5 min at 100 °C. Reaction in the pH range of 2—5 was carried out in 0.05 M acetate buffer. For pH 6—7 and 8—12, reaction was done in 0.05 M phosphate buffer and Tris–HCl buffer, respectively. The 100% value is the activity under standard assay conditions.

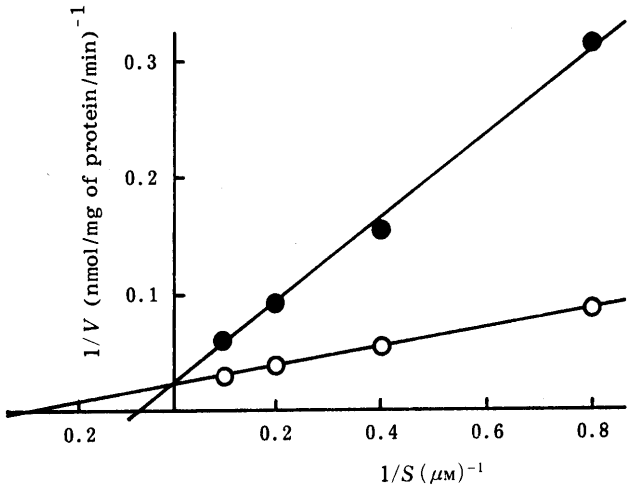

Fig. 4. Lineweaver–Burk Plots of Acid CEH in the Presence (●) or Absence (○) of Heat Treated Bovine Serum Albumin (0.8 μg)

Standard assay conditions are as described in Materials and Methods.

Effect of Albumin Heated at 100 °C on Acid CEH Activity Figure 2 shows the effect of heat treatment of albumin at 100 °C, pH 7.0 on its capacity to inhibit acid CEH activity. The albumin-dependent inhibition of acid CEH activity was markedly increased by heat treatment of the albumin, and the degree of enhancement was dependent upon treatment time. Figure 3 shows the inhibitory effect on acid CEH activity of albumin heated at various pH values. The stimulatory effect of heat treatment became stronger with elevation of pH in the range of 2.0—12.0, and disappeared at pH 2.0.

Kinetic Studies The kinetics of inhibition of acid CEH by heat-treated albumin are shown as Lineweaver–Burk plots in Fig. 4. This type of inhibition was found to be competitive.

Effect of Sodium Deoxycholate on Acid CEH Inhibition by Albumin We next tested the effect of sodium deoxycholate on the heat-treated albumin-dependent inhibition of acid CEH activity. The addition of sodium deoxycholate at a concentration above or below the inhibitory concentration did not restore the activity of acid CEH (data not shown).

Effects of Various Albumins on Acid CEH Activity We then tested the inhibitory effects on acid CEH activity of various albumin types, such as those from human, bovine and rabbit, and found no significant differences in these effects among albumin types (data not shown). In addition, these albumins also produced any increment in the inhibition of acid CEH activity with heat treatment (data not shown).

Effect of Heat Treatment of Binding of HABA to Albumin It was reported previously that the spectrum of the probe dye HABA (2-(4-hydroxy phenylazo)-benzoic acid) shows a new band at about 480 nm upon binding with albumin.[18] Using the native albumin and albumin heat-treated at 100 °C for 5 min in buffer at pH 11.5, the absorbance of HABA at 480 nm was measured. As shown in Fig. 5, the heat-treated albumin appeared to have no ability to enhance the absorbance of HABA at 480 nm.

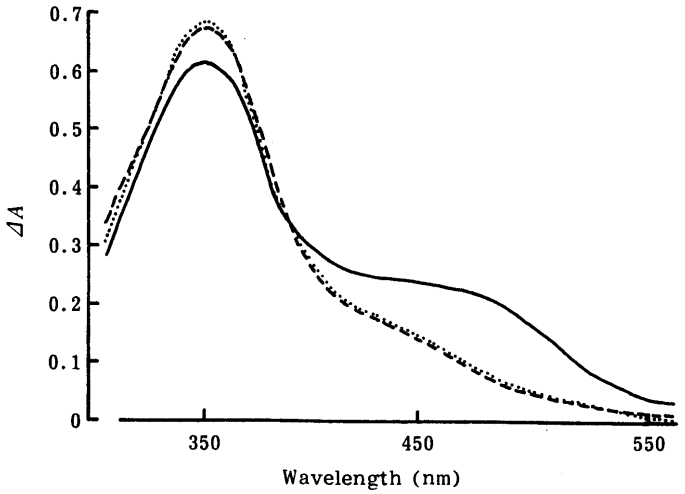

Fig. 5. Effect of Heat Treatment on Binding of HABA to Bovine Serum Albumin

Albumin heated at 100 °C for 5 min in 0.05 M Tris–HCl buffer at pH 11.5. The solution was dialyzed against 0.05 M phosphate buffer at pH 7.0. Spectra were taken at 25 °C. Sample solution: 5×10^{-5} M HABA in the presence of 2×10^{-5} M native bovine serum albumin (——) or 5×10^{-5} M HABA in the presence of 2×10^{-5} M heat-treated bovine serum albumin (------). Reference solution: 5×10^{-5} M HABA (---).

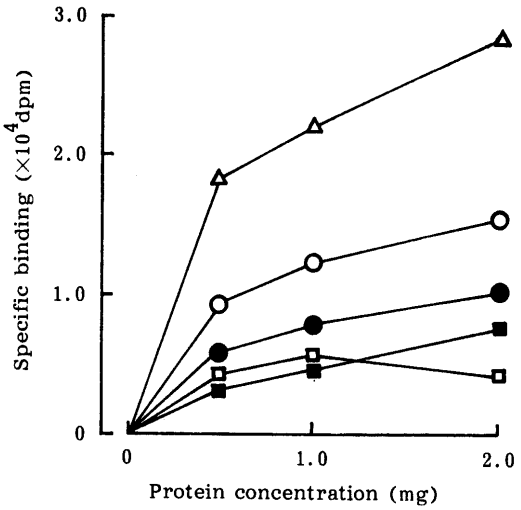

Fig. 7. Binding of Modified Albumins to Cholesteryl Oleate Droplets

Modified albumins were incubated for 10 min with cholesteryl (1-C^{14}) oleate droplets. The reaction was stopped by adding 5% trichloroacetic acid and the solution mixed for 30 s and centrifuged for 10 min at 3000 rpm. The amount of radioisotope in the pellet was measured. Each point represents the mean of triplicate measurements. ○, native albumin; ●, albumin heated at 100 °C for 5 min; ■, acetyl albumin; □, succinyl albumin; △, methyl albumin.

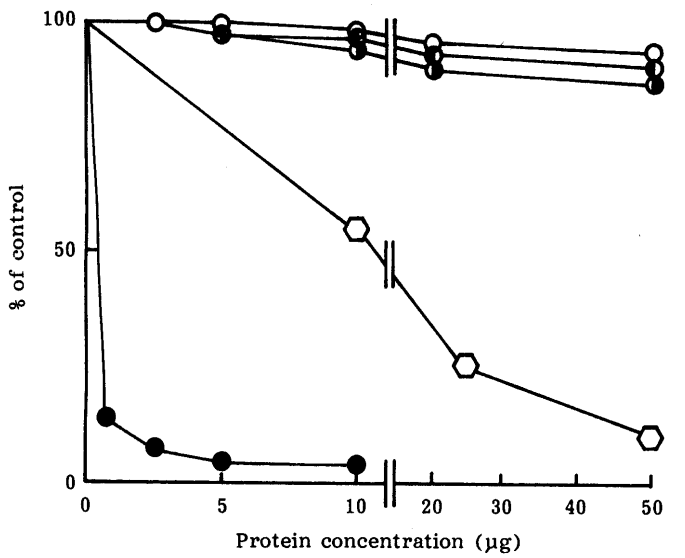

Fig. 6. Influence of Modified Albumin on Acid CEH Activity

The 100% value is the activity under standard assay conditions. ○, native bovine serum albumin; ◑, acetyl bovine serum albumin; ◐, succinyl bovine serum albumin; ●, methyl bovine serum albumin; ○, polylysine.

Effect of Various Modified Albumins on Acid CEH Activity For further investigation of the heat-induced enhancement of albumin-dependent inhibition of acid CEH activity, various modified albumins, such as methyl albumin, acetyl albumin and succinyl albumin were examined. As shown in Fig. 6, the acid CEH activity was markedly inhibited by albumin methylation, addition of about 0.6 μg causing approximately half-maximal inhibition. In contrast, the inhibitory effects on acid CEH activity by modified albumins, except methyl albumin, were much lower than that of albumin, and polylysine had no effect on the enzyme activity (Fig. 6). Moreover, no stimulatory effects of heat treatment on these modified albumins were observed (data not shown).

Binding of Modified Albumins to Cholesteryl Oleate Droplets To determine whether differences in the inhibitory effects of modified albumins were the consequences of differences in the reaction between modified albumins and the substrate, the affinity binding of modified albumins to the substrate was studied. As shown in Fig. 7, the affinity binding of modified albumins to the substrate became significantly weaker with heat-treated albumin, acetylalbumin and succinyl albumin and stronger with methyl albumin compared with albumin.

Discussion

It has been demonstrated that an inhibitor of lysosomal acid CEH in rat liver is present in the HDL and $d=1.21$ bottom fractions of rat serum, and that the inhibitor in the HDL fraction is apo A—I.[11] Here, the inhibitory effects of the $d=1.21$ bottom fraction on acid CEH activity were examined. The $d=1.21$ bottom fraction contains many kinds of protein, most of which have been classified as albumin. As shown in Fig. 1, the $d=1.21$ bottom fraction and albumin produced similar inhibitory effects with increasing protein concentration. These results suggest that one component of the inhibitory effect of the $d=1.21$ bottom fraction on acid CEH activity may be due to albumin itself.

On the other hand, there have been several studies on changes in the solubility and sedimentation velocity of albumin denatured by prolonged heating at various pH values.[19,20] With heat treatment at 80 °C in strong alkaline solutions, native albumin is denatured completely into monomer, dimer and higher aggregates.[21] In addition, from studies with the probe dye HABA, Terada *et al.*[18] reported that HABA shows a new spectral band at about 480 nm with native and heat-resistant albumin, but not with denatured albumin. In this study, we demonstrated that the albumin-dependent inhibition of acid CEH activity was markedly increased by heat treatment, and that this effect was progressively enhanced with prolonged treatment time at high pH. These results suggest that the enhancement effect of heat treatment on the albumin-dependent inhibition

of acid CEH activity is due to denaturation of albumin, and that this effect becomes stronger at high pH, probably because the exchange of disulfide bonds associated with albumin denaturation is greater at a high OH concentration.[18,21,22] However, it is not clear at the present whether more than one component may be responsible for the inhibitory activity. In addition, chemical modification of proteins with carboxylic or dicarboxylic anhydride and *O*-methylisourea has been used successfully to investigate the biological role of amino groups and to introduce different functional groups.[23-25] In the present study, the albumin-dependent inhibition of acid CEH activity was significantly increased by methylation of the albumin, but decreased by acetylation and succinylation. These results suggest that the stimulatory effects of heat treatment or methylation on the albumin-dependent inhibition may be due to consequent changes in the affinity and conformation of albumin. On the other hand, it has been reported that the loss of positive changes due to blockage of lysyl residues such as acetylation, succinylation and methylation gives to the particle a higher anionic electrophoretic mobility.[14,15] In addition, polylysine has a kationic electrophoretic mobility. It is therefore suggested that the inhibiting effects of modified albumins are the consequences of a change of the affinity binding of modified albumins to the substrate. However, the results presented here show that these inhibitory effects are not due to a significant difference in the affinity binding to the substrate. They suggest that the inhibitory effects of heat-treated albumin or methyl albumin may not lead to a change in the electrophoretic mobility of albumins or to the interaction between albumins and the substrate. However, the biological role of amino groups in the albumin-dependent inhibition of acid CEH activity is not clear, because the conformational implications of such modification have not yet been established experimentally.

Much information has been accumulated to suggest that biological or chemical modification of LDL converts it to a form recognizable by macrophages, leading to greatly enhanced cellular uptake and promotion of cholesteryl ester accumulation.[26-31] Moreover, formaldehyde-treated albumin is taken up and subsequently degraded in lysosomes by sinusoidal liver cells.[32,33] In this study, the albumin-dependent inhibition of acid CEH activity was markedly increased by chemical modification by heat treatment or methylation. From this information, it appears that denatured albumin may inhibit the acid CEH activity of macrophages. However, there is no evidence for the presence of a denatured form of albumin that can inhibit the activity of acid CEH in biological systems. In addition, it is not yet clear whether denatured albumin can inhibit acid CEH activity in its lysosomal environment.

On the other hand, because of the insolubility of cholesteryl esters in aqueous systems, the substrate preparations used for assay of acid CEH have been shown by several authors to affect the enzymal activity.[34-36] Klemets and Lundberg[37] reported that the interfacial concentration and molecular packing of substrate cholesteryl ester molecules in the droplet surface significantly affect the hydrolytic activity of acid CEH. In addition, Gargouri *et al.*[38] reported that amphiles such as proteins inhibited pancreatic lipase activity, and that this inhibition could be the result of desorption of lipase from its substrate due to a change in interfacial quality. Moreover, bile salts have been reported to increase neutral CEH activity in liver[39] and arterial tissues,[40] although high concentrations are inhibitory, but it is not clear at present whether bile salts affect the substrate, the enzyme or both in the enzymatic reaction. In this study, we demonstrated that the inhibitory effect of heat-treated albumin was not significantly different in the presence or absence of sodium deoxycholate at low and high concentrations. Albumin competitively inhibited the hydrolytic activity of acid CEH on cholesteryl esters. However, it is not clear at present whether heat-treated albumin affects the enzymatic reaction by its impact on the substrate interface, the enzyme interface or both.

References

1) T. G. Warner, L. M. Dambach, J. H. Shin, and J. S. O Brien, *J. Biol. Chem.*, **256**, 2952 (1981).
2) J. C. Dousset, N. Dousset, M. J. Foglietti, and L. Douste-Blazy, *Biochim. Biophys. Acta*, **664**, 273 (1981).
3) T. Imanaka, K. Muto, S. Ohkuma, and T. Takano, *FEBS Lett.*, **137**, 115 (1982).
4) K. T. Stokke, *Biochim. Biophys. Acta*, **270**, 156 (1972).
5) R. Klemets and B. Lundberg, *Lipids*, **19**, 692 (1984).
6) H. R. Sloan and D. S. Fredrickson, "In Metabolic Basis of Inherited Disease," 3rd ed., ed by J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, McGraw-Hill, New York, 1972, pp. 808—832.
7) J. L. Goldstein, S. E. Dana, J. R. Faust, A. L. Beaudet, and M. S. Brown, *J. Biol. Chem.*, **250**, 8487 (1975).
8) D. M. Small, *N. Engl. J. Med.*, **297**, 873 (1977).
9) R. Subbiah and B. Dicke, *Atherosclerosis*, **24**, 575 (1976).
10) D. Kritchevsky and H. V. Kothari, *Biochim. Biophys. Acta*, **326**, 489 (1973).
11) M. Tanaka, T. Iio, and T. Tabata, *Lipids*, **25**, 775 (1990).
12) P. Brecher, J. Chobanian, D. M. Small, and A. V. Chobanian, *J. Lipid Res.*, **17**, 239 (1976).
13) M. Tanaka, T. Iio, and T. Tabata, *Lipids*, **23**, 126 (1988).
14) A. A. Ansari, S. A. Kidwai, and A. Salahuddin, *J. Biol. Chem.*, **250**, 1625 (1975).
15) S. A. Kidwai, A. A. Ansari, and A. Salahuddin, *Biochem. J.*, **155**, 171 (1976).
16) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).
17) T. T. Hatch and R. S. Lees, *Adv. Lipid Res.*, **6**, 1 (1968).
18) H. Terada, K. Aoki, and M. Kamada, *Biochim. Biophys. Acta*, **342**, 41 (1974).
19) M. Levy and R. C. Warner, *J. Phys. Chem.*, **58**, 106 (1954).
20) R. C. Warner and M. Levy, *J. Am. Chem. Soc.*, **80**, 5735 (1958).
21) K. Aoki, K. Sato, S. Nagaoka, M. Kamada, and K. Hiramatsu, *Biochim. Biophys. Acta*, **328**, 323 (1973).
22) L. O. Andersson, *Biochim. Biophys. Acta*, **200**, 363 (1970).
23) G. Di Prisco, B. D. Udine, M. Pepe, and F. Scalenzhe, "In Chemical Reactivity and Biological Role of Functional Groups in Enzymes," ed. by, R. S. Smellie, Academic Press, New York, 1970, pp. 107—146.
24) A. F. S. A. Habeeb, *Arch. Biochem. Biophys.*, **121**, 652 (1967).
25) C. Jacobsen, L. Funding, N. P. H. Moller, and J. Steensgaard, *Eur. J. Biochem.*, **30**, 392 (1972).
26) A. Faggiotto, R. Ross, and L. Harker, *Arterioclerosis*, **4**, 341 (1984).
27) M. E. Rosenfeld, T. Tsukada, A. Chait, E. L. Bierman, A. M. Gown, and R. Ross, *Arteriosclerosis*, **7**, 24 (1987).
28) J. L. Goldstein, Y. K. Ho, S. K. Basu, and M. S. Brown, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 333 (1979).
29) M. S. Brown and J. L. Goldstein, *Annu. Rev. Biochem.*, **52**, 223 (1983).
30) R. E. Morton, G. A. West, and H. F. Hoff. *J. Lipid Res.*, **27**, 1124 (1986).
31) W. Palinski, M. E. Rosenfeld, S. Yla-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 1372 (1989).
32) M. Nilsson and J. Berg, *Biochim. Biophys. Acta*, **497**, 171 (1977).
33) J. L. Mego, "In Lysosomes in Biology and Pathology," ed. by J. T. Dingle, North Holland Publishing Co., Amsterdam, London, 1973, pp. 138—168.

34) P. Brecher, H. Y. Pyun, and A. V. Chobanian, *J. Lipid Res.*, **18**, 154 (1977).
35) T. Takano and T. Imanaka, *Acta Histochem. Cytochem.*, **11**, 323 (1978).
36) C. De Duve, *J. Lipid Res.*, **21**, 961 (1980).
37) R. Klemets and B. Lundberg, *Lipids*, **21**, 481 (1986).
38) R. Gargouri, R. Julien, A. Sugihara, R. Verger, and L. Sarda, *Biochim. Biophys. Acta*, **795**, 326 (1984).
39) K. T. Stokke, *Biochim. Biophys. Acta*, **270**, 156 (1972).
40) T. Takano, W. J. Black, T. J. Peters, and C. De Duve, *J. Biol. Chem.*, **249**, 6732 (1974).