Biochimica et Biophysica Acta, 1029 (1990) 161–166
Elsevier

BBAMEM 75024

Antioxidant activity of probucol and its effects on phase transitions in phosphatidylcholine liposomes

Larry R. McLean and Karen A. Hagaman

Merrell Dow Research Institute, Cincinnati, OH (U.S.A.)

(Received 30 April 1990)

Key words: Fluorescence polarization; Dimyristoylphosphatidylcholine; DSC

The effect of probucol on the phase behavior of dimyristoylphosphatidylcholine (DMPC) was examined by fluorescence polarization and differential scanning calorimetry (DSC). Probucol broadens and shifts the temperature of the main phase transition of DMPC liposomes as measured by fluorescence polarization with diphenylhexatriene and trimethylammonium-diphenylhexatrine at concentrations as low as 5 mole%. As measured by DSC, probucol reduces the transition temperature of the gel → liquid-crystalline phase transition of DMPC by approx. 2 C° at all concentrations above about 5 mole% probucol and eliminates the pretransition at < 1 mole%. In addition, the phase transition of DMPC is broadened and the enthalpy of the transition reduced by approx. 50%. Even at high concentrations of probucol, the gel → liquid-crystalline phase transition of DMPC is not eliminated. Similar effects are observed with dipalmitoylphosphatidylcholine liposomes. Based on these DSC measurements, measurements of the melting of probucol in dry mixtures with DMPC and observations of probucol mixtures with DMPC under polarizing optics, the maximum solubility of probucol in DMPC is approx. 10 mole%. This concentration exceeds that required (approx. 0.5 mole%) to prevent peroxidation of 10 mole% arachidonic acid in DMPC liposomes for 30 min in the presence of 0.05 mM $Fe(NH_4)(SO_4)_2$ at 4 °C. Thus, probucol has a limited solubility in saturated phosphatidylcholine bilayers, but is an effective antioxidant at concentrations lower than its maximum solubility.

Introduction

Probucol, 4,4′-isopropylidenedithiobis(2,6-di-*t*-butylphenol), is effective in reducing plasma cholesterol concentrations in animals [1] and in patients with hypercholesterolemia [2–5]. It prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits [6,7] and reduces achilles tendon xanthomata in humans [8]. The mechanism of action of probucol appears, at least in part, to be due to its properties as an antioxidant [9,10]. It has been proposed that probucol limits oxidative modification of low density lipoproteins (LDL), which have been observed in vivo [11], and thus minimizes uptake of modified LDL by macrophages preventing the lipid-loading which may result in production of foam cells in atherosclerotic lesions [12]. In addition, probucol reduces the rate of uptake of acetylated-LDL by macrophages under some experimental conditions [13] but not others [6,17,14], suggesting a possible effect on the macrophage cell membrane independent of its antioxidant activity. Probucol has also been proposed to alter the catabolism of LDL [15,16], decrease hepatic synthesis of lipoproteins [17,18], interfere with the assembly of proteins and lipids [19] and inhibit the secretion of interleukin-1 [20].

Several of these alterations may be the result of altered lipoprotein structure. Although the relationship between metabolic changes and purely structural changes is not clear, the interaction of probucol with the surface lipid components of lipoproteins is of considerable interest. In pure phosphatidylcholines, probucol markedly reduces the rate of association of soluble apolipoproteins, particularly at temperatures above the phase transition temperature of the lipid [19]. This effect is not observed with cholesterol at equivalent concentrations, so that it is not simply due to the presence of hydrophobic molecules within the bilayer. These observations suggest that probucol may play a structural role in addition to its role as an antioxidant.

Probucol is particularly effective in preventing peroxidative damage to the surface of low-density lipoproteins (LDL) [21]. This inhibition is due in part to the antioxidant effectiveness of probucol, but may also be related to its effects on lipid structure in the lipoprotein.

Correspondence: L.R. McLean, Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, OH 45215, U.S.A.

Probucol does not directly alter the structure of apoB-100 of LDL [21], but rather protects the structure under conditions of peroxidative stress. However, probucol does not completely prevent peroxidation of LDL lipids, even though it completely protects LDL surface structure [21]. This suggests that probucol prevents a secondary event which, in its absence, results in modification of lysine groups on the surface of LDL that alter its interaction with receptors. One possibility for this additional effect lies in prevention of the transfer of peroxidatively damaged lipids to the surface of the LDL, confining the reaction of the peroxidized lipids to internal lysines. For these reasons, which suggest a possible important structural role for probucol which contributes to its antiatherogenecity, the physical effects of probucol on the major lipid class of cell membranes was examined. In the present report, phase transitions of phosphatidylcholines are studied to examine the effects of probucol on the physical structure of the primary lipid component of biological membranes and their relationship to the antioxidant properties of this hypocholesterolemic agent.

Methods

Liposomes were prepared in 10 mM Tris-HCl (pH 7.4 at 24°C; pH 8.0 at 4°C; pH 7.1 at 37°C) buffer as described previously [19]. Polar lipids were obtained from Avanti Polar Lipids (Birmingham, AL), fluorescent probes were from Molecular Probes (Eugene, OR) and all other chemicals were of ACS reagent grade or better.

For peroxidation experiments, liposomes were prepared containing 10 mole% arachidonic acid (Sigma) and various concentrations of probucol; the remaining lipid was dimyristoylphosphatidylcholine (DMPC). Samples were diluted to 0.5 mg DMPC/ml and preequilibrated for 1–2 h at 4°C. An aliquot of 0.5 ml was taken for initial measurement of thiobarbituric acid reactive substances (TBARs). The samples were adjusted to 0.05 mM $Fe(NH_4)(SO_4)_2$ and 0.5 mM ascorbic acid by addition of aliquots of fresh solutions of the salts in standard buffer. Aliquots were taken at intervals for measurement of TBARs. The TBARs were measured by addition of 0.5 ml of sample to 0.1 ml of butylated hydroxytoluene (2%), followed by 1.5 ml each of 20% trichloroacetic acid and 0.67% thiobarbituric acid/0.05 M NaOH. Reaction proceeded for 30 min at 100°C. The tubes were then cooled, centrifuged for 15 min at 3000 rpm and transferred to plastic semimicro cuvettes. The difference in absorbance at 532 nm and 580 nm (to correct for light scattering) was measured in a Beckman DU-7 spectrophotometer. The TBARs were calculated in units of malondialdehyde equivalents using a molar extinction coefficient of $1.56 \cdot 10^5\ M^{-1} \cdot cm^{-1}$.

The fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH), were added to liposomes in a 1 : 200 molar ratio (probe : PC) in a volume of less than 10 μl of trifluoroethanol. The samples were then incubated for 1 h at 24°C prior to fluorescence measurements. Polarization measurements were made with Glan-Thompson polarizers on an SLM 4800 in the T-format. The ratio of the parallel to horizontal emission signals was measured with the excitation polarizer oriented vertically (R_v) and horizontally (R_h). The anisotropy is given by $((R_v/R_h) - 1)/((R_v/R_h + 2)$. Temperatures were regulated with an external water bath to ±0.2 C°.

For differential scanning calorimetric (DSC) measurements, liposomes were stored under N_2 for 24–48 h at room temperature prior to use. DSC of liposomes was performed on a Microcal MC-2 differential scanning calorimeter operating at a scan rate of 20 C°/h. Sample concentrations were 0.1 to 0.2 mg/ml. The differential voltage signal from the thermopiles, the temperature of the heat sink and the time were recorded at 10-s intervals on an IBM-PC-AT computer. The data were converted to cal/g per C° after dividing by the scan rate and the weight of sample. Enthalpies were calculated by numerical integration of the area under the transition; the phase transition temperatures (T_m) correspond to the temperatures at which the heat capacities reach maximum values during the transitions. Onset and end temperatures were measured at the intersection of a line drawn through the transition curve to the extrapolated baseline [22].

For dry mixtures of DMPC and probucol, samples were scanned at a rate of 5 or 10 C°/min in a Perkin-Elmer DSC-7. Scans were digitized with SigmaScan software and converted to a format suitable for analysis with the MC-2 software in a BASIC program. Hot stage observations were made with a Nikon Microphot-FX microscope equipped with a 10X eye-piece and 10X objective and polarizing optics. Temperature was regulated with a Mettler FP-52 heating stage and FP-5 controller. The samples were equilibrated for 2–3 days at −20°C and then scanned at 5 to 10 C°/min. At intervals, scanning was stopped and the samples were examined and photographed under crossed polarizers.

Results

The antioxidant activity of probucol in DMPC liposomes was assessed by measuring its effect on the rate of peroxidation of arachidonic acid in DMPC liposomes. This mixture was chosen to minimize perturbation of the liposome structure by the oxidizable lipid. As has been shown by Cervato et al. [23], at temperatures above the phase transition temperature of the liposomes, the rate of peroxidation of arachidonic acid is slow. At 37°C for DMPC liposomes containing 10

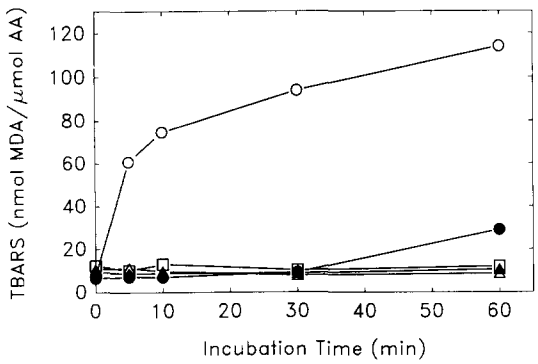

Fig. 1. Peroxidation of arachidonic acid in DMPC liposomes containing various concentrations of probucol at 4° C. (○) DMPC alone, no probucol; (●) 1% probucol; (△) 5% probucol; (■) 10% probucol; (□) 20% probucol.

mole% arachidonic acid, the TBARs are < 5 nmol MDA/μmol arachidonic acid after 1 h of incubation in the presence or absence of probucol. At a temperature below the phase transition temperature (4°C), arachidonic acid is rapidly peroxidized in the absence of probucol (Fig. 1). At 4°C, probucol was effective at concentrations as low as 0.5 mole% (Fig. 2). At concentrations of 1 mole% probucol, the rate of peroxidation is decreased to immeasurable levels over a period of at least 30 min. At higher probucol concentrations, arachidonic acid was protected from peroxidation for at least 1 h.

The effect of probucol on phase transitions in DMPC was examined by fluorescence polarization of bilayer probes and differential scanning calorimetry. Probucol broadens the phase transition of DMPC as measured by DPH and TMA-DPH probes in DMPC bilayers (Fig. 3). However, little effect of probucol on DPH polarization values either above or below the phase transition temperature is observed. The main phase transition temperature (T_m) of DMPC as measured by DPH polarization is decreased by about 4 C° at concentrations as small as 5 mole% probucol. At higher concentrations (20 mole%) only mimimal further broad-

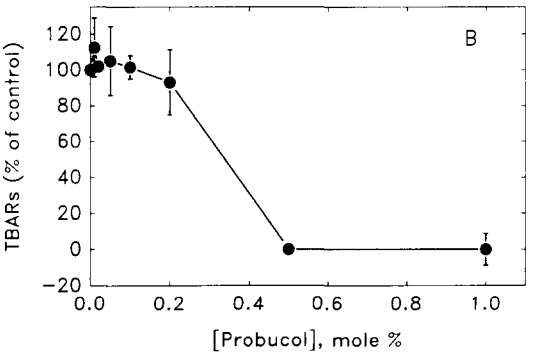

Fig. 2. Concentration-dependence of inhibition of lipid peroxidation by probucol. Samples of DMPC containing 10 mole% arachidonic acid and various concentrations of probucol were peroxidized with Fe^{2+}-ascorbic acid and TBARs were measured after 30 min of incubation at 4° C.

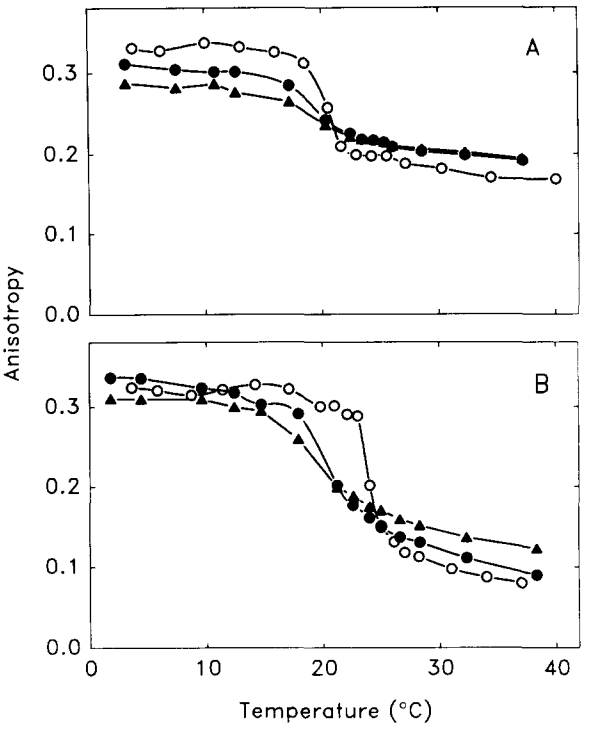

Fig. 3. Fluorescence anisotropy of (A) TMA-DPH or (B) DPH in DMPC liposomes without (○) and with probucol at a concentration of 5 mole% (●) and 20 mole% (△).

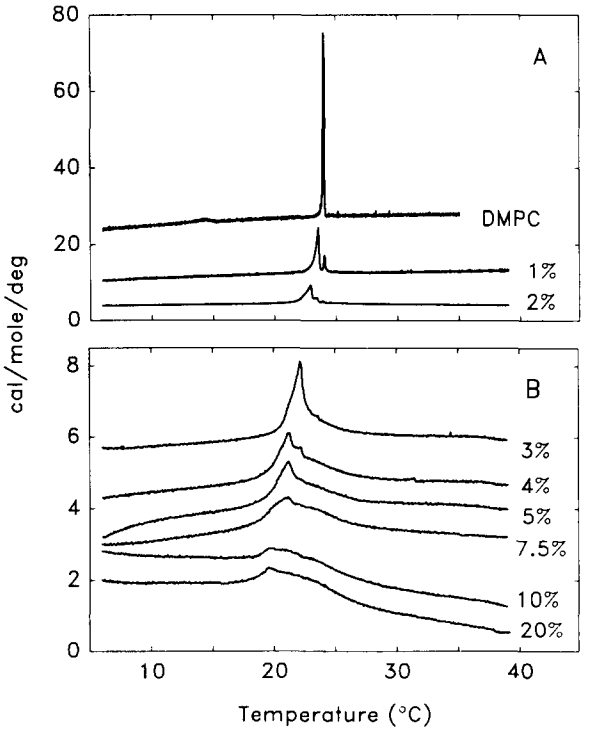

Fig. 4. Differential scanning calorimetric scans of probucol in DMPC liposomes. The per cent values refer to the mole% of probucol in DMPC.

ening or shifts in the transition temperature are observed. The phase transition in the head group region (measured by TMA-DPH) is considerably broadened by probucol and this effect increases with increasing probucol concentrations at temperatures below T_m.

The effect of probucol on the thermodynamics of the phase transition of DMPC was investigated by DSC. Probucol broadens the main phase transition of DMPC bilayers (Fig. 4) and eliminates the pretransition at concentrations of probucol < 1 mole%. The phase transition temperature is reduced by about 2 C°. Similar effects were observed with DPPC. The thermodynamic data for the main phase transition of DMPC in the presence of probucol is presented in Fig. 5. The effect of probucol on the enthalpy of the gel → liquid-crystalline phase transition in DPPC is also shown. It is evident that probucol exerts its maximum effects at all concentrations exceeding ≈ 5 mole% and does not completely eliminate the gel → liquid-crystalline phase transition at any concentration in either lipid.

The solubility of probucol in phosphatidylcholine was determined by measuring the phase transition of probucol in DMPC liposomes. At 10 mole% probucol, no probucol transition was detectable in dry mixtures of DMPC and probucol in the range 100–140°C. Phase

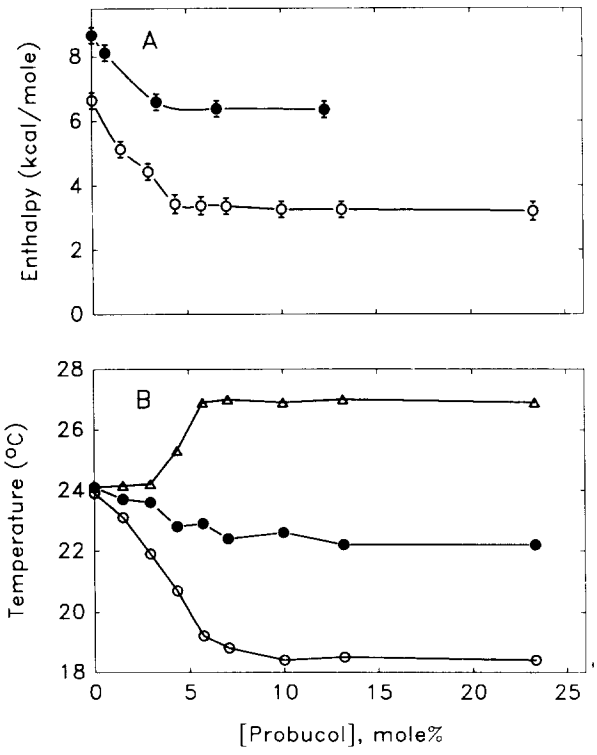

Fig. 5. Effect of probucol on the thermodynamic parameters of the phase transition of DMPC. (A) Enthalpy of the main phase transition of DPPC (●) and DMPC (○) liposomes as a function of probucol concentration. (B) Main phase transition onset temperature (○), midpoint temperature (●) and endpoint temperature (△) for probucol in DMPC liposomes.

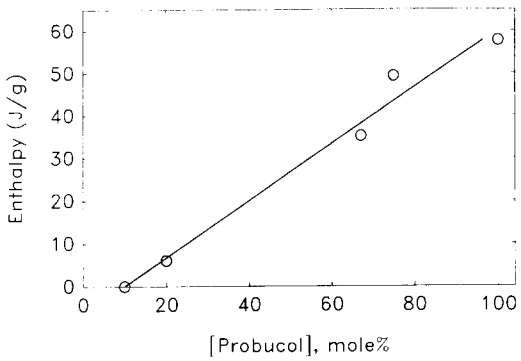

Fig. 6. Enthalpy of probucol transitions as a function of added probucol concentration based on DSC scans of dry samples of probucol in DMPC.

transitions were observed commencing with a concentration of about 20 mole% probucol (Fig. 6). Pure probucol forms rectangular crystals which melt irreversibly at 127 °C in the hot stage. When observed under polarizing optics, no crystal melting corresponding to probucol was observed in samples containing 10 mole% probucol. In 20 mole% probucol samples, some crystal melting was observed which was complete at about 135°C. These observations are consistent with the loss of enthalpy of the melting of probucol in the presence of increasing concentrations of DMPC, in which no free melting probucol is observed at concentrations below about 10 mole% probucol.

Discussion

The present report focuses on the effects of probucol on the structure of phosphatidylcholine bilayers and its potential as an antioxidant. Based on both DSC and fluorescence polarization results, probucol broadens and shifts the temperature of the main phase transition of DMPC liposomes at concentrations as low as 5 mole%. In dry mixtures with DMPC, DSC measurements of the melting of probucol are consistent with a maximum solubility of approx. 10 mole% in DMPC. Observations with polarizing optics confirm that the probucol melt observed by DSC is due to macroscopic phase separation of probucol at concentrations in excess of 10 mole% and not due to microscopic phase separation into domains within the bilayer. By contrast, only approx. 0.5 mole% probucol is required to prevent peroxidation of arachidonic acid in DMPC liposomes. Thus, the probucol concentration required for physical effects on the bilayer structure is somewhat greater than that which blocks lipid peroxidation in a model membrane bilayer. In addition, although probucol has a limited solubility in phosphatidylcholine bilayers, it is effective as an antioxidant at concentrations much lower than its maximum solubility.

Some information on the possible location of probucol within the bilayer may be obtained from examining the effect of probucol on the shape of the DSC scans. In general, a broadening and a decrease in enthalpy of the phase transition is observed with increasing probucol concentrations up to 10 mole%. These data are consistent with a location of probucol in a region of the bilayer in which the cooperativity of the acyl chain melting is disrupted and argue against an interbilayer location of the antioxidant. Two types of effects on the shape of the endotherm are observed. At low probucol concentrations (< 3 mole%), the area and height of the main transition decrease with little change in half-height width and a new peak appears as a shoulder on the low temperature side of the main transition. The half-height width and area of the new peak increases with increasing probucol concentrations (type B↓ [24]). This suggests an effect of probucol on the glycerol backbone region of the bilayer at low probucol concentrations. Such an effect on the surface region of the bilayer is also supported by the fluorescence polarization data. At higher concentrations of probucol the phase transition measured by DSC broadens and decreases in temperature (type A↓) as expected for membrane perturbants that partition into the C_1–C_8 methylene regions of the bilayer [24]. These data are consistent with a model in which probucol extends from the C_1–C_8 methylene region into the glycerol backbone region of the bilayer. This location would result in the observed loss of cooperativity of the phase transition and the effects of probucol on the head-group region of the bilayer.

The solubility data show that the interaction of probucol with phosphatidylcholines differs substantially from that of α-tocopherol. In mixtures of α-tocopherol with phosphatidylcholines, the enthalpy of the phase transition of the lipid continues to decrease up to a α-tocopherol concentration of 25 mole% at which concentration no phase transition is observed [25]. For α-tocopherol, the onset and midpoint temperatures of the thermal transition are lowered with little effect on the completion temperature. This is consistent with preferential partitioning of the antioxidant into the fatty acyl chain region of the bilayer in the liquid-crystalline phase. These data suggest that α-tocopherol and not probucol may compete with cholesterol for sites within the membrane. Thus, the α-tocopherol concentration in a membrane may be limited by the ratio of α-tocopherol to cholesterol and not by the solubility of the α-tocopherol. By contrast, if probucol does not compete for cholesterol sites, its concentration in the membrane should depend primarily upon its solubility. Based on data with patients treated with probucol for hypercholesterolemia, the distribution of probucol is similar to the overall distribution of lipids in plasma [5]. The average concentration of probucol after 6 months of therapy is 0.04–0.08 mg/ml [4,5] which corresponds to 1–3% of the plasma phospholipid concentration by weight. Thus, probucol concentrations in plasma are near saturation based on the concentration of phospholipid. In experiments in which inhibition of LDL peroxidation was observed, the concentration of probucol was 5–10 mole% based on surface lipid [21]. In addition, the differing locations of probucol and α-tocopherol in the bilayer suggest that their sites of effectiveness may differ. Thus the physical properties of probucol and α-tocopherol may make these two antioxidants suited to the treatment of different types of disorders related to peroxidative damage.

Acknowledgements

We thank Dr. Ruth Homan for the use of her DSC and hot stage observations.

References

1 Barnhart, J.W., Rytter, D.J. and Molello, J.A. (1977) Lipids 12, 29–33.
2 Cortese, C., Marenah, C.B., Miller, N.E. and Lewis, B. (1982) Atherosclerosis 44, 319–325.
3 Miettinen, T.A., Huttunen, J.K., Naukkarinen, V., Strandberg, T. and Vanhanen, H. (1986) Am. J. Cardiol. 57, 49H–54H.
4 Fellin, R., Gasparotto, A., Valerio, G., Baiocchi, M.R., Padrini, R., Lamon, S., Vitale, E., Baggio, G. and Crepaldi, G. (1986) Atherosclerosis 59, 47–56.
5 Dachet, C., Jacotot, B. and Buxtorf, J.C. (1985) Atherosclerosis 58, 261–268.
6 Kita, T., Nagano, Y., Yokode, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H. and Kawai, C. (1987) Proc. Natl. Acad. Sci. USA 84, 5928–5931.
7 Carew, T.E., Schwenke, D.C. and Steinberg, D. (1987) Proc. Natl. Acad. Sci. USA 84, 7725–7729.
8 Yamamoto, A., Matxuzawa, Y., Yokoyama, S., Funahashi, T., Yamamura, T. and Kishino, B.-I. (1986) Am. J. Cardiol. 57, 29H–35H.
9 Parthasarathy, S., Young, S.G., Witztum, J.L., Pittman, R.C. and Steinberg, D. (1986) J. Clin. Invest. 77, 641–644.
10 Jackson, R.L., McLean, L.R., Akeson, A L., Ku, G., Barnhart, J.W. and Mao, S.J.T. (1990) in Xth International Symposium on Drugs Affecting Lipid Metabolism, Elsevier/North-Holland, Amsterdam, in press.
11 Palinski, W., Rosenfeld, M.E., Ylä-Herttuala, S., Gurtner, G.C., Socher, S.S., Butler, S.W., Parthasarathy, S., Carew, T.E., Steinberg, D. and Witztum, J.L. (1989) Proc. Natl. Acad. Sci. USA 86, 1372–1376.
12 Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) New Engl. J. Med. 320,915–924.
13 Yamamoto, A., Takaichi, S., Hara, H., Nishikawa, O., Yokoyama, S., Yamamura, T. and Yamaguchi, T. (1986) Atherosclerosis 62, 209–217.
14 Ku, G., Schroeder, K, Schmidt, L.F., Jackson, R.L. and Doherty, N.S. (1990) Atherosclerosis 80, 191–197.
15 Nestel, P.J. and Billington, T. (1981) Atherosclerosis 38, 203–209.
16 Kesaniemi, Y.A. and Grundy, S.M. (1984) J. Lipid Res. 25, 780–790.
17 Li, J.R., Holets, R.J. and Kottke, B.A. (1980) Atherosclerosis 36, 559–565.
18 Balasubramaniam, S., Beins, D.M. and Simons, L.A. (1981) Clin. Sci. 61, 615–619.

19 McLean, L.R. and Hagaman, A. (1988) Biochim. Biophys. Acta 959, 201–205.
20 Ku, G., Doherty, N.S., Wolos, J.A. and Jackson, R.L. (1988) Am. J. Cardiol. 62, 77B–81B.
21 McLean, L.R. and Hagaman, K.A. (1989) Biochemistry 28, 321–327.
22 Phillips, M.C., Ladbrooke, B.D. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35–44.
23 Cervato, G., Viani, P., Masserini, M., DiIorio, C. and Cestaro, B. (1988) Chem. Phys. Lipids 49, 135–139.
24 Jain, M.K and Wu, N.M. (1977) J. Membrane Biol. 34, 157–201.
25 Massey, J.B., She, H.S. and Pownall, H.J. (1982) Biochem. Biophys. Res. Commun. 106, 842–847.