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Biochemistry

Oxygen-mediated heterogeneity of apo-low-density lipoprotein

(lipid oxidation/free radicals/protein fluorescence/molecular weight/sodium dodecyl sulfate/polyacrylamide gel electrophoresis)

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ABSTRACT **Mild oxidation of human serum low-density lipoprotein (LDL) converts the apoprotein from a nearly homogeneous component of high apparent molecular weight to a mixture of apparently lower molecular weight polypeptide components, as characterized by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. This protein alteration, which correlates temporally with increases in the formation of lipid oxidation products and in the fluorescence of the apoprotein, is markedly reduced when oxygen is excluded or when EDTA or the free-radical-scavenging antioxidants, butylated hydroxytoluene or propyl gallate, are added. The conversion thus appears to be due to a reaction between the protein moiety and auto-oxidizing lipid. The presence of the antibacterial agent sodium azide markedly accelerates the oxidation, suggesting that it should only be used with caution in lipid-containing solutions.**

Structural and functional characterization of serum lipoproteins has received attention because molecular or metabolic abnormalities associated with this class of molecules might be significant in the development of atherosclerosis (1). The low-density lipoprotein fraction (LDL) has been of particular interest because of its apparent role in atherogenesis. Such a role is indicated by a correlation between high serum levels of LDL and clinical atherosclerosis (2), as well as the accelerated atherosclerosis in patients who have an inherited abnormality in LDL catabolism (familial hypercholesterolemia) (3). LDL is catabolized after it is bound through its protein moiety (apo-B or apo-LDL) to a cell-surface receptor (4).

Although characterization of the apoprotein of LDL has been difficult (5), studies on the lipid moiety appear to be less controversial. LDL contains a large proportion of unsaturated lipids (somewhat variable on diet) (6) and is unique among the serum lipoproteins in its susceptibility for undergoing auto-oxidation *in vitro* (7–9). This finding has led some authors to speculate that such auto-oxidation plays a role in atherogenesis (7, 10, 11). Although structural (7–13), spectrophotometric (9, 13, 14), and lipid compositional (8, 11) changes in LDL have been observed upon oxidation, its polypeptide chain has not been characterized. Model studies with lipid–protein mixtures have shown that oxidized lipid can lead to major modification of protein components; such modifications include crosslinking (15), polypeptide scission (16), and loss of amino acids (17, 18). It thus seems possible that some of the difficulties encountered in characterizing apo-B are associated with partial oxidation of LDL during purification and/or storage. The observation that plasma lipids from human subjects contain significant diene conjugation (an early manifestation of lipid auto-oxidation) suggests that oxidation of LDL can be initiated *in vivo* (19).

We show here that mild oxidation of LDL *in vitro* results in altering the sodium dodecyl sulfate ($NaDodSO_4$)–apoprotein electrophoretic pattern from a single major band of low mobility into numerous bands of significantly higher mobility.

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MATERIALS AND METHODS

Lipoprotein Preparation. Fresh human serum was obtained from healthy adult male volunteers with normal serum cholesterol and triglyceride levels and normal lipoprotein electrophoretic patterns. Lipoproteins were purified from this serum after addition of disodium EDTA (final concentration, 0.05% wt/vol) and addition of the bacterial inhibitors, sodium azide, chloramphenicol, and merthiolate, to final concentrations of 0.05%, 0.005%, and 0.005%, respectively (wt/vol). Approximately 3-ml aliquots of serum were placed in centrifuge tubes and each was overlayered with 1 ml of 10 mM Na_2 phosphate-buffered saline, pH 7.4. After centrifugation at 16° in a Beckman SW56 rotor for 18 hr at 40,000 rpm, the top 0.5 ml containing the very-low-density lipoprotein (VLDL) fraction was removed. A concentrated NaCl solution was then added to the infranatant to obtain a final salt concentration of 1.72 molal. Subsequent centrifugation as above for 23 hr gave the LDL fraction at the top. Purity of the LDL was established by electron microscopy, lipid analysis by thin-layer chromatography, LDL electrophoresis in polyacrylamide gels, and polyacrylamide electrophoresis of the $NaDodSO_4$–protein complex. The last method is particularly useful in demonstrating polypeptide chains characteristic of possible contaminants such as VLDL, high-density lipoproteins, and serum albumin when electrophoresis is done under overloading conditions. No such contaminants were detected.

Lipoprotein Oxidation. The purified lipoprotein, diluted to about 1 mg of protein per ml, was dialyzed for 24 hr at 4° with numerous changes of a solution of phosphate-buffered saline (pH 7.4) containing the bacterial inhibitors used in the original preparation. Oxidation was then allowed to proceed in a loosely covered flask at room temperature by continued dialysis against buffer saturated with air.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Aliquots of the dialyzing lipoprotein were removed at various times during dialysis; the $NaDodSO_4$–apo-B complex was formed either with apoprotein obtained after organic solvent delipidation at −10° (20) in the presence of 0.001% butylated hydroxytoluene (21) or by direct addition of excess $NaDodSO_4$ to lipoprotein to a final concentration of 1.5%. After $NaDodSO_4$ addition, 2-mercaptoethanol was added to a concentration of 1% and the solutions were heated at 100° for 2 min. Both methods for $NaDodSO_4$–protein complex formation gave the same electrophoretic results, in agreement with previous work (22, 23). Dialysis of the sample against the ionic medium of the electrophoresis buffer to eliminate ion fronts that might potentially disturb electrophoretic migration gave similar results. The samples were frozen and then run simultaneously after reheating at 100° in the presence of 2-mercaptoethanol. No change in the protein electrophoretic pattern was found due to the freezing. Electrophoresis was conducted in a continuous buffer system containing 30 mM sodium borate/30 mM sodium

Abbreviations: LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate.

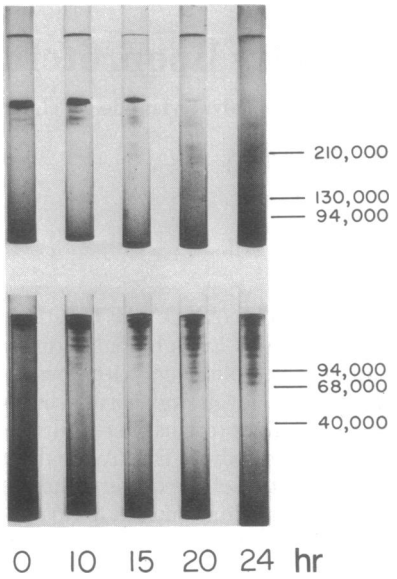

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of apo-B (30 μg) after dialysis of LDL under oxidizing conditions for various lengths of time. Duplicate samples were run on (*Upper*) 2.6% and (*Lower*) 7.0% gels. Standard proteins used for reference (with their molecular weights) were myosin (210,000), β-galactosidase (130,000), phosphorylase (94,000), bovine serum albumin (68,000), and aldolase (40,000).

acetate/0.1% $NaDodSO_4$ at pH 8.5 and using gels with a total acrylamide concentration of either 2.6% or 7.0% (0.14% as *N,N'*-methylenebisacrylamide).

After electrophoresis at 1–3 mA/gel for 5 hr, the gels were removed, fixed in a solution containing 30% methanol/8% acetic acid, and then stained with Coomassie blue. After destaining by diffusion, the protein bands were evaluated quantitatively by densitometry at 575 nm by a Corning 740 System scanner with automatic integration.

Thiobarbituric Acid Reaction. Concentrated trichloroacetic acid was added to aliquots of lipoprotein samples containing 1.5 mg of protein to give a final concentration of 5%. An equal volume of 1% thiobarbituric acid was then added and the mixture was heated in a water bath at 100° for 20 min. After centrifugation to clarify the solution, the peak absorbance at 532 nm was read on a Beckman DB spectrophotometer against a buffer blank. The amount of thiobarbituric-reactive substance was calculated from a standard curve, with malonaldehyde bis(dimethylacetal) (Aldrich) as the standard (24).

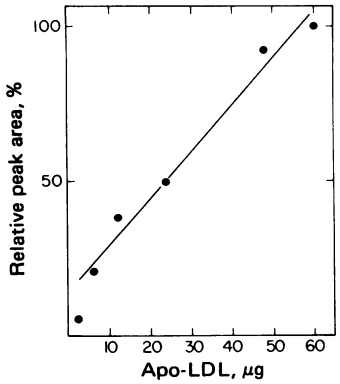

FIG. 2. Relationship between amount of intact apo-B (zero-time) applied to 2.6% gel and peak area from densitometric scan of Coomassie blue-stained band after electrophoresis. Results are normalized with respect to the peak area of the highest concentration applied.

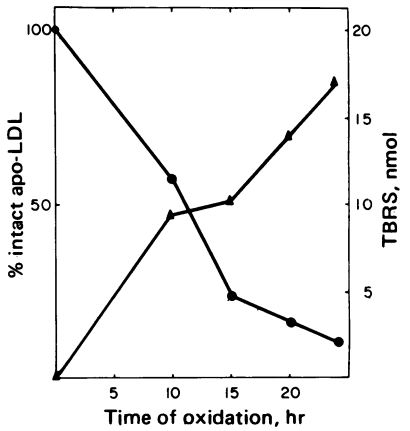

FIG. 3. Temporal relation of apo-B conversion with lipid oxidation as determined by formation of thiobarbituric acid reactive substances (TBRS). ●, Apo-B; ▲, TBRS.

Fluorescence. Fluorescence spectra were determined with an Aminco-Bowman spectrophotofluorimeter on aliquots of apoprotein that had been delipidated by organic solvents (20, 21) and then solubilized in $NaDodSO_4$.

Apparent Molecular Weight Estimation. Unoxidized apo-B was prepared by organic solvent delipidation, solubilized in $NaDodSO_4$, and then dialyzed against the electrophoresis buffer. Standard proteins of known molecular weight were treated similarly. Electrophoresis of the proteins, both separately and combined, was done simultaneously on 3.5% polyacrylamide gels (12 cm) at 1 mA/gel for 8.5 hr. The buffer solutions were recirculated by a peristaltic pump. Mobility was evaluated after staining from the densitometric scans.

RESULTS

When LDL is dialyzed against air-saturated buffer, the apoprotein pattern obtained after $NaDodSO_4$/polyacrylamide gel electrophoresis on 2.6% and 7.0% gels shows a conversion with time from a band of low mobility to multiple species with more rapid migration rates (Fig. 1). All of the protein entered the 2.6% gel, but larger species were excluded from the 7% gel. A minor band in the undialyzed sample appears to be an early product of the conversion since it increases with length of dialysis. The conversion was evaluated quantitatively for the amount of the major species on the 2.6% gel by densitometric scans, and the area under the resulting curve was normalized to percent of the zero-time value. A nearly linear relation was obtained in standard curves for the amount of apo-B present in the concentration range examined (Fig. 2).

The decrease in intact apo-B corresponds to the formation of lipid oxidation products that, at early times of oxidation, can be monitored by an increase in absorbance due to these products reacting with thiobarbituric acid (25, 26) (Fig. 3). The conversion is prevented, however, if nitrogen is bubbled through the dialysis solution (Fig. 4), indicating a requirement for oxygen. It is also inhibited in air when EDTA is added (Fig. 5). A likely explanation of this result is that divalent metal ions, which accelerate LDL oxidation (10), are contaminating the buffer in small amounts. The addition of either a lipid-soluble free-radical-scavenging antioxidant, butylated hydroxytoluene (Fig. 6), or a water-soluble one, propyl gallate (Fig. 7), also inhibits the conversion, as would be expected if free radicals participated in the protein alteration. The presence of sodium azide in the dialysate accelerated the conversion considerably (Fig. 8). No effect was seen when the serine protease inhibitor phenylmethyl sulfonyl fluoride was added at a concentration

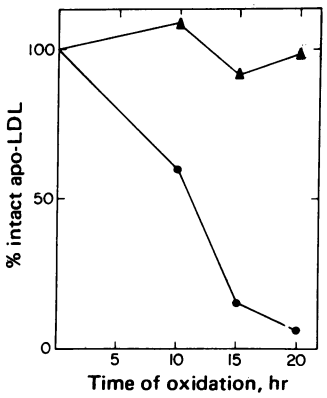

FIG. 4. Effect on apo-B conversion when the dialysis solution is equilibrated with N_2 (▲) compared to an air-oxidized control (●).

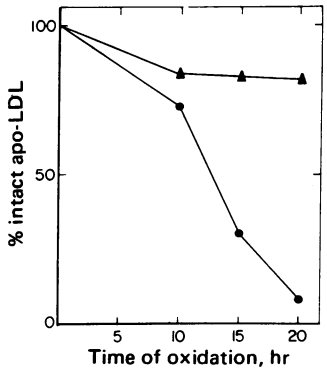

FIG. 6. Inhibition of apo-B conversion by 2 mM butylated hydroxytoluene. A concentrated ethanol solution of butylated hydroxytoluene was added to the lipoprotein prior to dialysis. ●, Control; ▲, 2 mM butylated hydroxytoluene present.

that inhibited 100% of the activity of a solution of trypsin (34 units/ml). The apo-B present in VLDL is converted more slowly than that in LDL (Fig. 9).

In addition to the change in the mobility of the apoprotein, an increase in its fluorescence correlated with the time of oxidation (Fig. 10). The increase occurs with an excitation maximum of 370 nm and an emission maximum of 450 nm, similar to the spectrum of lipofuscin or "age" pigments found *in vivo*, which are believed to be substituted 1-amino-3-iminopropene structures, formed when auto-oxidized lipids react with protein (15, 27, 28).

The electrophoretic mobility on $NaDodSO_4$/polyacrylamide gel electrophoresis for the major zero-time protein band of apo-B was compared by extrapolation with that of other proteins of known molecular weight. The mobility of apo-B corresponds to that which would be expected for a typical $NaDodSO_4$-protein complex with a molecular weight of 4.0×10^5 (Fig. 11). This "apparent molecular weight" serves to describe the behavior of unoxidized apo-B under well-defined experimental conditions.

DISCUSSION

The conditions used for dialysis, under which the observed protein conversion occurs, promote lipid oxidation (8, 10). The temporal correlation of the formation of thiobarbituric-reactive substance with the protein conversion (Fig. 3) suggests an interrelationship between the oxidation of lipid and the observed changes in the protein. VLDL, which shows a slower protein conversion than LDL (Fig. 9), has also been found by others to be less sensitive to oxidation (7, 8). Furthermore, the inhibitory effectors of protein conversion found here all inhibit lipid oxidation (Figs. 4–7). We thus conclude that the protein conversion is due to a chemical interaction between protein and oxidizing lipid. The observed reproducible appearance of specific components during this conversion may result from an increased susceptibility to oxidation of unique amino acid sequences or from differential lipid binding along the length of the apoprotein.

Studies on the oxidation of lipid–protein mixtures have demonstrated that one type of effect on the protein is the covalent binding of lipid oxidation products, some of which can result in fluorescent substituents, including crosslinks (15, 27, 28). This reaction also occurs in our system, as shown by the increase in protein fluorescence with increasing time of oxidation (Fig. 10). Another observed effect on protein in the presence of oxidizing lipid is peptide scission (16). Although the mechanism for this is not known, it may involve the formation of protein free radicals, known to be formed by reaction with oxidizing lipid (29). The destruction of protein amino acids in oxidized lipid–protein mixtures (17) increases with time in the same manner as the production of free radicals (18). That protein conversion is inhibited by free-radical-scavenging antioxidants (Figs. 6 and 7) provides evidence for a role for free radicals in producing the changes seen here. Either of these effects, scission or covalent binding of lipid oxidation products, could result in protein alteration. The comparatively low apparent molecular weights observed for long-term oxidation products and the fluorescence results suggest that both may, in fact, be occurring.

Alternatively, the hypothesis that apo-B is composed of multiple chains covalently linked by oxidizable, unusual lipid or carbohydrate bonds would also be consistent with the data.

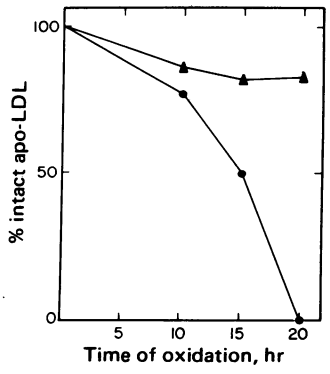

FIG. 5. Protection of apo-B against conversion when 1 mM EDTA is included in the dialysis solution. ●, Control; ▲, 1 mM EDTA added.

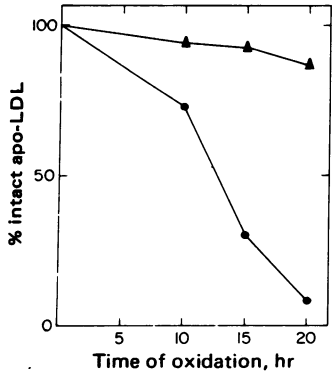

FIG. 7. Effect on apo-B conversion when 5 mM propyl gallate is included in the dialysis solution. ●, Control; ▲, 5 mM propyl gallate added.

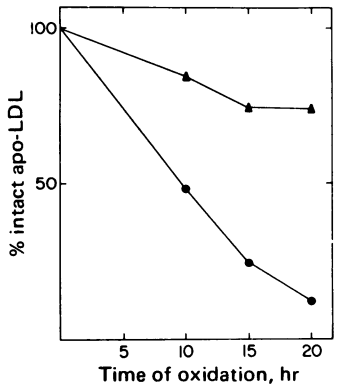

FIG. 8. Stimulation of apo-B conversion by sodium azide. ●, Control; ▲, control minus sodium azide.

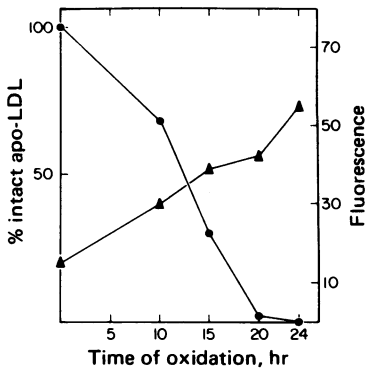

FIG. 10. Temporal relation of apo-B conversion with acquired fluorescence. ●, Apo-B; ▲, fluorescence (relative intensity with excitation at 370 nm and emission at 450 nm).

Covalent modification of amino acid residues by lipid oxidation products might also interfere with unusually strong noncovalent bonding domains between any hypothesized subunits. However, we prefer the speculation that polypeptide cleavage is responsible, since the alternate mechanisms appear too *ad hoc* and are without precedent.

Among the isolated lipoprotein fractions, LDL is most sensitive to oxidation (7–9). In agreement with these observations, we find that the apo-B in VLDL is altered less rapidly than that in LDL (Fig. 9). This result may be due to differences in the lipid components of these two lipoproteins since the rate at which lipids oxidize depends on the degree to which they are unsaturated. The major lipid species in LDL is cholesterol ester (5), which is formed through a reaction in which lecithin-cholesterol acyltransferase catalyzes transfer of the fatty acid side chain from the β position of lecithin to cholesterol (30). This fatty acid is preferentially unsaturated due to the substrate specificity of the phospholipid acyltransferase (31). Thus, the LDL core contains unsaturated esterified fatty acids in the range of 35–70%, depending on diet (6). In contrast, the corresponding unsaturated fatty acid content of the major VLDL lipid component, triglycerides, is only 7–45% (6). Another factor that may contribute to the more rapid oxidation of LDL compared to VLDL could result from differences in the physical state of the lipid phase, which is considerably less mobile in LDL (32, 33).

The interesting observation that sodium azide promotes LDL oxidation (Fig. 8) suggests that its use as an antibacterial agent should be avoided in studies on systems containing lipid. The mechanism of this effect may be related to the observation that sodium azide in solution increases the inhibitory effect of pulse radiolysis on yeast alcohol dehydrogenase through formation of an N_3 radical (34). The auto-oxidizing lipid in our system may act on the azide in a manner similar to that seen for irradiation, since previous studies have shown that oxidizing lipids exhibit radiomimetic effects (35).

Gel electrophoresis of the $NaDodSO_4$–apo-B complex from unoxidized VLDL and LDL repeatedly resulted in a sharp major band of very low mobility. Comparison of this mobility with that of $NaDodSO_4$–protein complexes of known molecular weight indicates an apparent molecular weight of 4.0×10^5. If apo-B were a typical protein whose molecular weight could be reliably determined by this empirical method, that value would represent the upper limit possible, since the apo-B mass per lipoprotein molecule is reported to remain constant at about 3.7×10^5 g/mole during the metabolic conversion of VLDL to LDL (36). However, $NaDodSO_4$/polyacrylamide gel electrophoresis is not a primary method for determining molecular weight and does not guarantee that $NaDodSO_4$–apo-B behaves in a typical manner. Inasmuch as artifactual deviations from ideal or expected behavior have not been the subject of this study, definitive conclusions from these data regarding the true molecular weight of apo-B remain as yet unwarranted.

During this study, we noted that one or more minor components were always observed after gel electrophoresis of the $NaDodSO_4$–apo-B complex not subjected to oxidation *in vitro*. The mobilities of these bands correspond to that of early *in vitro* oxidation products. Since these minor bands were obtained despite all attempts to inhibit oxidation during purification, the result is consistent with the hypothesis that LDL oxidation can be initiated *in vivo*, a possibility that has already been suggested

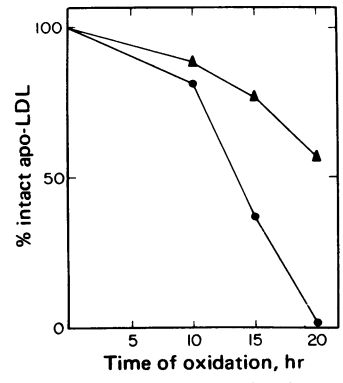

FIG. 9. Comparison of apo-B conversion from the LDL fraction and the VLDL fraction. ●, LDL; ▲, VLDL.

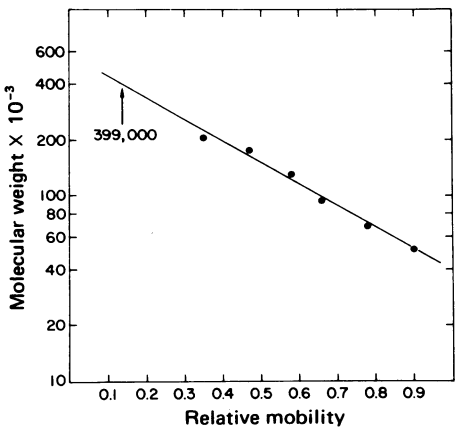

FIG. 11. Apparent molecular weight of intact (zero-time) apo-B as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis on a 3.5% gel. Standards used are myosin (210,000), bacterial glutamate synthetase (175,000 and 52,000), β-galactosidase (130,000), phosphorylase (94,000), and bovine serum albumin (68,000). Mobilities are expressed relative to that for lysozyme (14,000), which ran near the front.

on the basis of other data. For example, the report that human plasma lipids contain significant diene conjugation (19) is in accord with the occurrence of lipoprotein oxidation in the blood. In addition, diverse treatments that are expected to increase the potential for oxidation in blood decrease cholesterol levels in serum. Thus, the pro-oxidant effect of increased dietary polyunsaturated fats (37–39), intramuscular injection of peroxidase (40), increased oxygen respiration (41–43), and whole-body ultraviolet irradiation (44, 45) decreased serum cholesterol levels. In contrast, the expected anti-oxidant effect of increased dietary saturated fats (38, 39, 46), decreased oxygen respiration (47, 48), cigarette smoking (49), increased carbon monoxide respiration (50, 51), dietary vitamin E (52–54), and dietary butylated hydroxytoluene (55) resulted in increased levels of serum cholesterol. The report that LDL catabolism is decreased in cells cultured under hypoxic conditions (56) and the finding that oxidized steroids can mimic the effect catabolized LDL has on certain cellular activities (57) further support the hypothesis that oxidation plays a role in the clearance of LDL.

The studies reported here show that oxidation of LDL can occur under mild conditions with rather dramatic consequences on the apoprotein structure. The degree to which this phenomenon may have contributed to the divergent published results for the molecular weight of apo-B is uncertain, but care in future studies of this kind to limit LDL oxidation is clearly indicated. In addition, mechanisms that lead to the oxidative degradation of apo-B into artifactually discrete components may also occur in other lipid–protein systems (e.g., membranes). Caution must therefore be exercised in the interpretation of $NaDodSO_4$ band patterns obtained in such systems.

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