Cholesterol Behavior in Human Serum Lipoproteins†

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ABSTRACT: A derivative of ergosterol, ergosta-5,7,9,22-tetraen-3β-ol, was synthesized and characterized. Its properties in membranes are similar to those of cholesterol as measured by glucose permeability and by order parameters derived from electron spin resonance of spin-labels. Thus, because of the three conjugated double bonds, this molecule can be used as an optical probe of sterol behavior in membranes. Circular dichroism (CD) spectra of sonicated egg phosphatidylcholine vesicles containing the probe exhibited CD transitions whose intensity was dependent on sterol content. CD spectra from this probe in human low-density and high-density lipoproteins indicated distinctly different environments for the sterol in the two lipoproteins.

Human low-density lipoprotein (LDL)[1] and high-density lipoprotein (HDL) are two of the major carriers of cholesterol in human blood. Plasma levels of LDL have been suggested to have a direct correlation with the deposits of cholesterol in plaques on arterial walls and subsequent atherosclerosis (Fredrickson & Levy, 1972; Smith, 1974). Recently, plasma levels of HDL have been inversely correlated with the development of atherosclerosis, appearing to protect against the development of the disease (Miller & Miller, 1975; Rhoads et al., 1976). Differences in the structure of these lipoproteins and the behavior of cholesterol in LDL and HDL are therefore important areas of inquiry.

Recently a derivative of ergosterol with conjugated carbon–carbon double bonds, ergosta-5,7,9,22-tetraen-3β-ol (dehydroergosterol), was introduced as a probe of cholesterol behavior by using its fluorescent properties (Rojers et al., 1979). It was shown to be similar to cholesterol by two criteria, water permeability studies and cell growth studies (Rojers et al., 1979). Here this derivative is further characterized, and the capacity of dehydroergosterol to produce circular dichroism (CD) spectra is exploited to study sterol behavior in human LDL and HDL and in rabbit sarcoplasmic reticulum.

Materials and Methods

Ergosterol was obtained from Sigma. Egg phosphatidylcholine was purchased from Avanti Biochemicals, and cholesterol and mercuric acetate were obtained from Fisher. The 12-doxyl derivative of stearic acid was supplied by Syva. The coupled glucose assay (with $NADP^+$) prepackaged by Sigma was used to assay for glucose concentrations. Sepharose CL-6B was obtained from Pharmacia, and the antibodies were obtained from Miles Laboratories. Triton X-100 was supplied by Calbiochem. All buffers were thoroughly purged with nitrogen before use.

Absorption spectra were recorded on a Cary 17 spectrophotometer. For each absorption spectrum obtained with the probe, an equivalent sample without a probe was used in the reference compartment. Spectra were recorded at 23 °C. ESR spectra were recorded on a Varian E-9 spectrometer at 9.5 GHz at room temperature (23 °C) with a 100-G sweep width, 1-G modulation, and 20-mW microwave power. The stearic acid spin-label was incorporated at 1 mol % with respect to the phospholipids, and measurements were obtained with a 100-μL sample in an aqueous flat cell. Phospholipid concentrations at 10 mg/mL were employed. Order parameters, S, were calculated as described elsewhere (Griffith & Jost, 1976). Circular dichroism spectra were obtained on a Jasco J21-C spectropolarimeter at room temperature (23 °C) in 1-cm path-length cells. Samples for CD measurements exhibited an absorbance between 0.1 and 1 in the wavelength region scanned. For each preparation containing the dehydroergosterol from which a CD spectrum was obtained, a control CD spectrum was run on the same preparation without the dehydroergosterol. Fluorescence spectra were obtained on an SLM fluorescence instrument.

Preparation of Ergosta-5,7,9,22-tetraen-3β-ol (Dehydroergosterol). Ergosterol was recrystallized from methanol before use. All reactions were performed in the dark and in a glovebox under N_2 to prevent decomposition. Ergosterol was dehydrated with mercuric acetate in chloroform and acetic acid for 18 h, following previously published procedures (Ruyle et al., 1953). The acetate form of the product was recrystallized from ether and ethanol and exhibited a melting point of 168 °C. The free alcohol had a melting point of 143 °C. Both melting points are in agreement with previously published values (Zurcher et al., 1954). The near-UV absorption spectrum (250–350 nm) and the fluorescence spectrum from this material were identical with previously published spectra (Rojers et al., 1979).

Glucose Release. For preparation of liposomes for glucose release experiments, the appropriate amounts of egg phosphatidylcholine and sterol were dissolved in chloroform to ensure random mixing. The lipids were first dried under a stream of N_2 and then under vacuum. A glucose solution (0.3 M) was added and the tube vortexed to disperse the lipid. These liposomes were then diluted by a factor of 25 with a salt solution (0.075 M NaCl and 0.075 M KCl) and centrifuged in a Beckman L5-50B with a 50 rotor for 5 min at 49 000 rpm. This procedure was repeated twice to remove external glucose.

For measurement of glucose release, the vesicles were incubated for 1 h, and then one-half of the liposomes were rewashed as described above. The latter sample then contained only the glucose which had not leaked from the vesicles during the incubation. Glucose was liberated with the minimum Triton X-100 required to solubilize the liposomes, and the amount trapped before and after incubation was determined by the Sigma glucose assay. This procedure is a modification

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[1] Abbreviations: CD, circular dichroism; ESR, electron spin resonance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-1249$01.25/0

of one previously used (Demel et al., 1972).

Preparation of Samples for ESR and CD Measurements. The appropriate lipids were dried from chloroform as described above, with the addition of spin-labeled stearic acid in the ESR samples. The lipids were dispersed in 10 mM histidine, pH 7, by vortexing. For the ESR measurements, an egg phosphatidylcholine concentration of 10 mg/mL was used. Cholesterol at the indicated mole ratios was added when the lipids were mixed in chloroform.

For the CD measurements, the phospholipid concentration was 2 mg/mL. Furthermore, for the CD measurements, small sonicated vesicles were formed to reduce light scattering. The lipid dispersion was sonicated with a Branson 350 sonifier for three 5-min periods, with 2 min between each sonication, in an ice bath. The nearly clear solution was centrifuged at 45 000 rpm in a 50 rotor for 45 min, and the clear supernatent was collected for spectral measurements. All samples were used within 48 h. For short-term storage, the samples were held in the dark under N_2 at 4 °C.

Preparation of Human Serum Lipoproteins. Human low-density ($1.019 < d < 1.065$) and high-density ($1.065 < d < 1.21$) lipoproteins were prepared by serial centrifugation from the blood of several normal donors obtained after an overnight fast. Fresh blood was drawn into ACD solution and processed immediately. Erythrocytes were removed by centrifugation at 8000 rpm in a Sorvall SS-34 rotor for 15 min. The serum was then centrifuged at 40 000 rpm in a 50 rotor for 18 h at 18 °C. Very low-density lipoproteins were removed, and a reference sample was subsequently removed to verify the density. The density was adjusted to 1.065, and after another 18-h centrifugation at 18 °C, the LDL was removed, along with a reference aliquot to verify the density. Finally HDL was harvested after adjustment of the density to 1.21 and another 24-h centrifugation at 18 °C. Each lipoprotein preparation was then chromatographed on a Sepharose CL-6B column, monitored with a Gibson UV monitor at 280 nm. The peak corresponding to either HDL or LDL was pooled. The pooled peak was rechromatographed on the same column before use. This procedure further removed minor contaminants from the lipoprotein preparations. LDL and HDL samples were stored in brown bottles under N_2 at 4 °C.

Preparation of Sarcoplasmic Reticulum. Sarcoplasmic reticulum was isolated from the white hind leg muscles of New Zealand white rabbits according to the procedure of Eletr & Inesi (1972). ATPase activity was assayed in the presence and absence of Ca^{2+} at 37 °C, and activities obtained agreed with previously published preparations (Warren et al., 1974). $NaDodSO_4$–polyacrylamide gel electrophoresis on 10% gels showed the previously identified bands, including the most prominant which corresponds to the Ca^{2+},Mg^{2+}-ATPase of that membrane.

Introduction of Dehydroergosterol into Lipoproteins and Sarcoplasmic Reticulum. For introduction of dehydroergosterol into LDL and HDL, egg phosphatidylcholine, cholesterol, and dehydroergosterol were mixed in chloroform at a 5:2:1 mole ratio. The solution was dried under a stream of nitrogen and then under vacuum. A buffer containing 10 mM histidine, 150 mM NaCl, and 1 mM EDTA at pH 7 was added, and the lipid film was suspended by vortexing at a concentration of 5 mg/mL in phospholipid. For the lipoproteins, the liposomes were incubated with the lipoproteins (24 mg/mL protein) by using equal volumes of each for 1 h at 23 °C in the dark under N_2 with gentle stirring. The samples were centrifuged at 40 000 rpm in a 50 rotor for 5 min to remove the liposomes from the lipoprotein. For the CD measurements of the probe in sarcoplasmic reticulum, egg phosphatidylcholine was mixed with dehydroergosterol in a 1:1 mole ratio, and liposomes were made as described above. These were sonicated to clarity and centrifuged at 49 000 rpm for 30 min. The phospholipid vesicles in the clear supernatant were then diluted to 2 mg/mL in phospholipid. Equal volumes of the vesicles and a dispersion of sarcoplasmic reticulum (at 1.5 mg/mL in protein) were incubated at 23 °C for 2 h under N_2 in the dark with gentle stirring. The sarcoplasmic reticulum was separated from the vesicles by centrifugation at 40 000 rpm for 15 min. A modification of the method just described was employed as an alternate means of introducing the probe into the lipoproteins. Dehydroergosterol was first incorporated into human erythrocytes by incubation with sonicated vesicles. Then the probe was transferred to HDL and LDL by incubation of the lipoproteins with the loaded red cells. After centrifugation to remove the red cells, the lipoproteins were repurified on Sepharose CL-6B. The concentration of probe in all these samples was determined by UV difference spectroscopy, using samples without the probe as a reference. Protein was assayed by using the method of Lowry et al. (1951) and phosphate by the Bartlett assay (1959). Cholesterol was determined enzymatically (Allain et al., 1974).

Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed in tube gels in a water-jacketed Buchler electrophoresis apparatus maintained at 15 °C with a Fisher refrigerated water bath. A discontinuous system was employed by using a 3% stacking gel at pH 8.8 on top of a running gel of the indicated percent at pH 6.8. Gels were stained with Coomassie blue and scanned on a Gilford gel scanner at 550 nm.

Gel Double Diffusion with Antibodies to Human Serum Lipoproteins. Goat antibodies to human LDL, HDL, and whole human serum were used. Double diffusion was performed from wells in 0.75% agarose with pH 7.2 phosphate-buffered saline. Diffusion was permitted for 2 or 3 days before staining with Coomassie. Titrations of the antibody against the antigen were performed to find the correct dilutions for band formation. When the LDL and HDL preparations were tested, two different antigen dilutions were used for each test.

Results

Characterization of Lipoproteins. Chromatography on Sepharose CL-6B demonstrated the purity of the two classes of lipoproteins with respect to size. The column profile for LDL and HDL rechromatographed on this column after the initial isolation from a column run as described above showed a single peak, as reported previously (Yeagle et al., 1977a). No attempt was made here to subfractionate the LDL and HDL. Analysis of the density of the reference aliquot verified the density ranges quoted.

$NaDodSO_4$–polyacrylamide gel electrophoresis of the LDL (on 6% gels) and HDL apoproteins (on 10% gels) shows little cross-contamination of the preparations. Examination of the LDL and HDL by the gel double diffusion described above using goat anti-human LDL and HDL, as well as goat antisera to whole human serum, likewise indicated negligible cross-contamination of the preparations. LDL showed a single line against anti-LDL and no line against anti-HDL. The HDL showed a single line against anti-HDL but also showed a weak line against anti-LDL. All of the LDL and HDL preparations used in these experiments exhibited the above-listed characteristics.

The characteristics of HDL and LDL incubated with erythrocytes for the purpose of incorporation of the dehydroergosterol (see Materials and Methods) were examined after the incubation and compared to the original HDL and

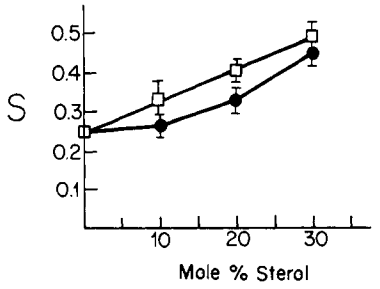

FIGURE 1: Order parameter, S, obtained from ESR measurements by using 12-doxyl stearate on egg phosphatidylcholine dispersions (2 mg/mL) containing several different mole percents of sterol. (□) Cholesterol; (●) dehydroergosterol.

LDL. Chromatography on Sepharose CL-6B demonstrated that neither lipoprotein fraction had changed detectably in size during the incubation. Phospholipid, cholesterol, and protein assays demonstrated that no significant change in the content of the HDL occurred during the incubation with erythrocytes. Similarly, analysis of the LDL showed no significant change in protein or cholesterol content and less than a 20% change in phospholipid content. $NaDodSO_4$ gel electrophoresis showed no change in the major peptides of either lipoprotein fraction during the incubation. Furthermore, analysis by thin-layer chromatography of the major phospholipids of the lipoproteins demonstrated that no significant changes took place in the phosphatidylcholine/sphingomyelin ratio during the incubation. Thus, the incubation procedure with erythrocytes did not cause any apparent change in the structure of the lipoproteins, which was expected since the lipoproteins in blood are constantly in contact with erythrocytes. Since the CD spectra from the lipoproteins incubated both ways were the same, no further characterization was performed.

Characterization of Dehydroergosterol. A variety of sterols have been characterized with respect to their effect on the permeability of phosphatidylcholine liposomes to glucose. Some sterols, with sufficient structural similarity to cholesterol, reduce glucose permeability as does cholesterol, while others either have no effect or else increase permeability (Demel et al., 1972; Yeagle et al., 1977b). Dehydroergosterol has been similarly characterized here. At 40 mol % sterol, liposomes of egg phoshatidylcholine with cholesterol retained 93 ± 2% of the original glucose after a 1-h incubation, while for dehydroergosterol 83 ± 2% was retained, indicating some kinship to cholesterol in behavior. A similar result was obtained with ergosterol (Demel et al., 1972). Without sterol, 73 ± 2% of the glucose was retained.

ESR spin-labels can be used to obtain motional order parameters for hydrocarbon chains in phospholipid bilayers. Cholesterol is known to increase the motional order of a membrane, a phenomenon reflected in the order parameters recorded in Figure 1. These data are obtained on unsonicated dispersions of egg phosphatidylcholine containing several different mole ratios of cholesterol. The data for dehydroergosterol show a similar, though not identical, behavior compared to cholesterol. While order parameters from ESR spin-labels have been shown to be somewhat perturbed compared to results from ^2H NMR, they are still useful for comparison of the behavior of these two sterols. Qualitatively the behavior of the order parameter is the same as that reported previously for cholesterol (Taylor & Smith, 1980).

Both these experimental approaches suggest similar, though not identical, behavior of dehydroergosterol and cholesterol. Elsewhere it has been shown that water permeability of membrances is affected similarly by both sterols and that dehydroergosterol is a suitable sterol to support the growth

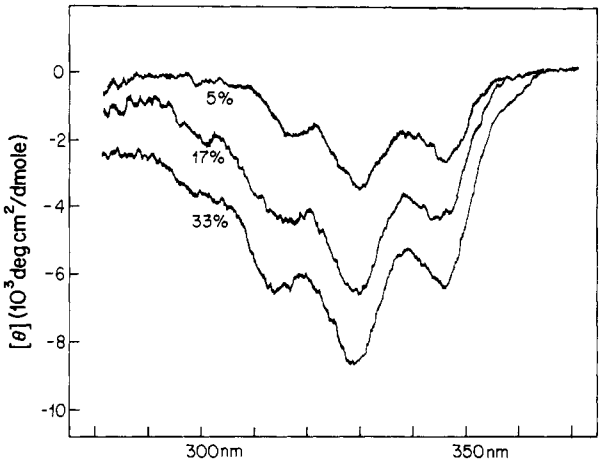

FIGURE 2: Circular dichroism spectra of dehydroergosterol in egg phosphatidylcholine vesicles containing the indicated mole percent of cholesterol and 6 mol % dehydroergosterol, measured in a 1-cm path-length cell.

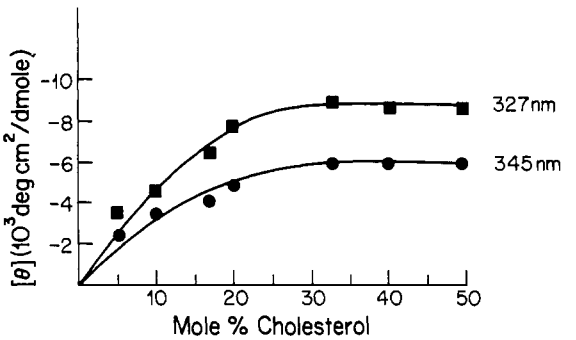

FIGURE 3: Magnitude of the CD spectra of dehydroergosterol in cholesterol-containing sonicated egg phosphatidylcholine vesicles at the indicated wavelength as a function of the mole percent of cholesterol in the vesicle.

of *Tetrahymena pyroformix* (Rojers et al., 1979). Since dehydroergosterol exhibits unique optical properties not exhibited by cholesterol, the similarity in behavior encourages the use of dehydroergosterol to probe cholesterol behavior.

Circular Dichroism of Dehydroergosterol in Vesicles. At low dehydroergosterol concentrations in egg phosphatidylcholine vesicles, no CD transitions were observed between 300 and 350 nm due to the dehydroergosterol. However, as is shown in Figure 2, the presence of cholesterol in the vesicle apparently induces CD transitions. The magnitude of these CD transitions is a function of the cholesterol concentration, as is seen in Figure 3. In these samples, the dehydroergosterol concentration was kept constant at a mole ratio of 1:11 with respect to the phospholipid. As described below, at this concentration no CD is seen from the probe by itself in the absence of additional sterol. As the mole fraction of cholesterol was then increased in these vesicles and the CD spectra were recorded, the spectra observed exhibited the same band shape seen in Figure 2, but with differing intensities when normalized for the amount of probe in the sample by using the UV absorption spectrum. The plot in Figure 3 reflects the change in magnitude of the CD at 327 and 345 nm. A graph of the same shape is obtained if the change in CD is monitored at any other wavelength between 300 and 350 nm. The behavior of the CD below 300 nm has not been explored due to interferences from proteins in the lipoprotein and membrane samples described below. Higher concentrations of cholesterol were not explored because this sonicated vesicle system is not well-defined at higher mole ratios of cholesterol (Newman & Huang, 1975).

Egg phosphatidylcholine vesicles containing only dehydroergosterol were also examined as a function of dehydroergosterol concentration. When high concentrations (30 mol %) of dehydroergosterol are in the vesicles, the same CD transitions are observed as in the vesicles just described which contain a small amount of dehydroergosterol and variable amounts of cholesterol. This is expected since both dehydroergosterol and cholesterol are sterols, and both can affect the motional ordering of the membrane.

At low dehydroergosterol concentrations (10 mol %), no CD transitions are observed between 300 and 350 nm. However, a positive transition has been noted in the region of 280–250 nm. This was not pursued due to interferences from protein CD bands in the protein-containing samples. Dehydroergosterol in $CHCl_3$ does not exhibit any readily detectable CD transitions.

Circular Dichroism of Dehydroergosterol in Human Serum Lipoproteins and Membranes. The previously described experiments give a picture of the kind of CD spectra one could expect from dehydroergosterol in phospholipid membranes with and without cholesterol. It was then reasonable to obtain CD spectra of dehydroergosterol in human LDL and HDL as a probe of cholesterol behavior in these lipoproteins. One likely location for cholesterol in LDL is in the monolayer of phospholipids felt by many to constitute part of the surface of LDL. Since the diameter of LDL is very close to the diameter of sonicated egg phosphatidylcholine vesicles (Huang, 1969; Forte & Nichols, 1972), the vesicle studies described above should provide a good model system, both because the radii of curvature of the outer surface of both assemblies is nearly the same and because the similar size would be expected to produce similar light-scattering artifacts. Similar arguments can be advanced for HDL, though less strongly since HDL is a smaller particle.

CD spectra were obtained from normal LDL and HDL and from LDL and HDL containing dehydroergosterol at 1 mol % with respect to the phospholipids. The probe was introduced into the lipoproteins as described under Materials and Methods. Since both LDL and HDL produce CD by themselves (Gotto et al., 1973; Chen et al., 1980), the spectra without the probe were subtracted from the spectra with the probe to obtain just the CD due to dehydroergosterol in these lipoproteins. These spectra were then normalized with the probe concentration derived from the UV absorption spectra. The results are shown in Figure 4. The CD spectra from LDL show the same transitions seen in the phospholipid vesicles containing cholesterol. Interestingly, however, the intensity of that spectrum is about that expected in an egg phosphatidylcholine vesicle with about 15 mol % cholesterol, as can be seen from a comparison of Figure 4 and Figure 3.

In contrast, dehydroergosterol in HDL produces CD spectra distinctly different from anything else that has been studied, including the membrane system cited below. Figure 4 shows two major transitions, similar to the two longer wavelength transitions seen in the other systems studied, but missing the two shorter wavelength transitions seen in all the other systems studied. Furthermore, the intensity is much less than that exhibited by dehydroergosterol in LDL.

For comparison purposes, the CD spectra of dehydroergosterol in rabbit muscle sarcoplasmic reticulum were also studied. The CD spectrum exhibited the same transitions with the same relative intensities among the transitions that are observed in egg phosphatidylcholine vesicles and in LDL.

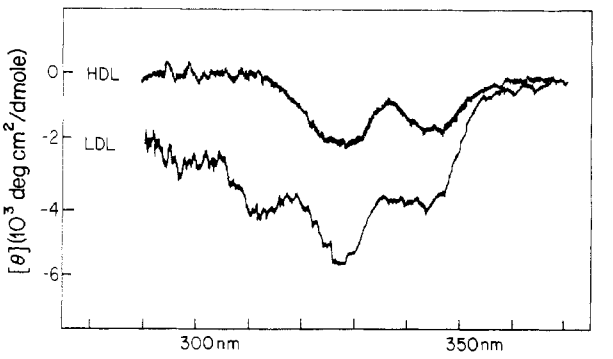

FIGURE 4: Circular dichroism spectra of dehydroergosterol in LDL and HDL, after subtracting the circular dichroism due to the lipoprotein without the dehydroergosterol. The probe concentration in the LDL sample was 2×10^{-5} M and in the HDL sample was 2.4×10^{-5} M. These are the spectra which result from the lipoproteins incubated with liposomes. Spectra from lipoproteins incubated with red cells are similar.

Discussion

The data presented here and elsewhere (Rojers et al., 1979) encourage the use of dehydroergosterol as a probe of cholesterol behavior in lipoproteins and in membranes. Substantial similarities have been noted in the effects of dehydroergosterol and cholesterol on glucose permeability and water permeability (Rojers et al., 1979). Similar increases in the ordering of the lipid hydrocarbon chains in the membrane are observed in the ESR data, which most likely reflect on glucose and water permeability. Further, at least one organism can use dehydroergosterol as a sterol substitute (Rojers et al., 1979). The results just enumerated suggest that the behavior of dehydroergosterol, which can be monitored by fluorescence (Rojers et al., 1979) and circular dichroism, can be used as a measure of cholesterol behavior.

The circular dichroism spectra arising from dehydroergosterol in sonicated vesicles are intriguing. At low dehydroergosterol concentrations and in the absence of cholesterol, no CD bands are observed. Only upon the addition of significant sterol components to the vesicles are any CD transitions observed. The band structure of the UV absorption spectrum is not similarly affected. The first question is then why sterol can induce these CD transitions.

A clue can be found in Figure 1. Cholesterol and ergosterol both increase the order parameter as measured with ESR. ^2H NMR likewise reports an increase in motional order in egg phosphatidylcholine membranes with increasing cholesterol content (Taylor & Smith, 1980). A decrease in the rate of axial rotation of cholesterol is also observed at higher cholesterol concentrations as shown in a recent ^{13}C NMR study (Yeagle, 1981). The effect plateaued as the amount of cholesterol exceeded 30 mol %, similar to the results reported here.

The differences in intensity of the CD spectra appear to reflect internal properties of the systems and not an artifact. Throughout the region of cholesterol concentration studied, the vesicles formed by sonication followed by column chromatograhy or centrifugation as used here (Barenholz et al., 1977) have been shown to be homogeneous with respect to size and composition (Newman & Huang, 1975). Only small changes in the vesicle radius are observed up to 33 mol % cholesterol (though the system is less well-defined at higher concentrations of cholesterol) (Newman & Huang, 1975). Thus, any distortions due to light scattering should be constant throughout these measurements. The same can be said for LDL, which is similar in size to these vesicles. No wavelength shifts were noted in the transition maxima of these CD spectra of dehydroergosterol.

The change in motional parameters noted above seems to provide the best explanation for the changes noted in the

circular dichroism spectra. As the sterol becomes more highly ordered and experiences slower axial rotation, the CD spectrum becomes more intense. Changes noted in the CD of proteins may provide a useful comparison. For some proteins, as they are cooled, an increase in CD intensity is observed (Strickland, 1974). This has been ascribed to the population of fewer conformers at lower temperatures and therefore a decrease in conformational mobility (Strickland, 1974). The protein is being "ordered", allowing for less movement between various conformations that would otherwise tend to cancel some of the CD observed. Thus, the ordering and slowing of the rate of rotation of dehydroergosterol in these egg phosphatidylcholine vesicles may be directly related to the increase in intensity of the observed CD bands. Ordering effects have also been noted in the induced CD of β-carotene in LDL, in which the CD was apparently sensitive to phase transitions in the core cholesterol esters (Chen et al., 1980).

With this background, it is appropriate to consider the CD from dehydroergosterol in LDL. The band structure of the CD spectra from dehydroergosterol, after subtracting the CD spectra due to the LDL, is the same as that observed from dehydroergosterol in phospholipid vesicles. This implies that the environment of the probe that is inducing the CD in the LDL is similar to the environment of the sterol in egg phosphatidylcholine vesicles, since CD is generally very sensitive to changes in environment. Such a result is reasonable in light of current models of LDL structure. The surface of the LDL is considered to consist of a monolayer of phospholipid surrounding a cholesterol–ester–triglyceride core (Tardieu et al., 1976; Shen et al., 1977; Yeagle et al., 1978). At least some of the cholesterol is felt to reside in that surface monolayer, which consists largely of phosphatidylcholine. Since that surface monolayer is much like the outer leaflet of a sonicated egg phosphatidylcholine vesicle, it is not surprising that the CD spectra of dehydroergosterol are similar in both systems. However, the CD intensity of the probe in the LDL is the same as that seen from the probe in sonicated egg phosphatidylcholine vesicles containing only about 15 mol % cholesterol. In LDL, free cholesterol is present in about a 1:1 mole ratio with the phospholipids. To the extent that a comparison between these two systems is valid, this result implies that much of the free cholesterol in LDL is not in the surface monolayer of the phospholipid. A finite solubility of cholesterol in a cholesterol–ester–triglyceride mixture has been reported (Deckelbaum et al., 1977; Small, 1970).

This implication is in remarkable agreement with deductions from a ^{31}P NMR study of the behavior of the phospholipids of LDL. It was shown that in LDL the phospholipid head group behaved as expected for a sonicated phospholipid vesicle with low cholesterol content, i.e., 10 mol % or less (Yeagle et al., 1978).

The above analysis applies to LDL incubated for a brief time with liposomes. LDL incubated with red cells shows a similar spectrum with the same relative intensity among the four transitions seen in Figure 4.

The CD spectra observed for dehydroergosterol in HDL incubated with red cells and with liposomes suggest a distinctly different environment for cholesterol in HDL compared to LDL or phospholipid vesicles. Two of the negative CD transitions seen in the latter two systems are not seen in the CD spectrum of dehydroergosterol in HDL, and the absolute intensity is less in HDL. HDL has been suggested to have an overall structure similar to that for LDL (Morrisett et al., 1975) with an outer monolayer of phospholipids (Assmann et al., 1974) surrounding a hydrophobic core. Only the protein component of the two lipoproteins is dramatically different.

One logical source for perturbations of the CD is the protein of HDL. From ^{13}C NMR studies, some of the free cholesterol is evidently in the core, but much of it is located in an environment with an undetermined but significantly reduced motional freedom (Avila et al., 1978). The latter environment could be next to protein. Preliminary results on the CD of dehydroergosterol bound to human erythrocyte glycophorin encourage such speculation. The spectal shape is similar to the HDL spectrum, suggesting sterol–protein interactions in HDL (P. L. Yeagle, unpublished results). CD spectra of small molecules, with conjugated double bonds, binding to water soluble proteins show pronounced effects of the environment of the protein on the CD transitions of the chromophores of the small molecules (Henkens & Sturtevant, 1972). Thus, it is reasonable to propose a sterol–protein interaction in HDL that is absent in LDL (which contains a distinctly different protein component) to explain the different CD results. The sterol–protein interaction may be important in the apparent protection HDL offers in the pathogenesis of atherosclerosis (Miller & Miller, 1975; Rhoads et al., 1976). It may also explain the more effective removal of cholesterol from macrophages by HDL than that observed with LDL (Ho et al., 1980).

A different sterol environment in these two lipoproteins may echo a difference previously detected in the phospholipid behavior in these two lipoproteins. About one-fifth of the phospholipid head groups in LDL were found to be immobilized by the intact B protein of LDL (Yeagle et al., 1977a). No corresponding phospholipid head-group–protein interactions were observed in HDL (Yeagle et al., 1978).

The above speculations on HDL, while seeming to be the best interpretation of the CD spectra of intact HDL, do not agree with interpretations of data obtained from HDL recombinants (Tall & Lange, 1978), in which cholesterol is suggested to be excluded from the lipid–protein interface. Presently, sufficient information is not available to reconcile these differences.

The CD spectra of dehydroergosterol in LDL, in sonicated phospholipid vesicles, and in the membrane of the rabbit sarcoplasmic reticulum, contain the same transitions. This is consistent with a sterol location among phospholipids engaging in little or no interaction with the major protein of the sarcoplasmic reticulum, the Ca^{2+},Mg^{2+}-ATPase, in agreement with activity studies which suggested that cholesterol is excluded from the immediate environment of that protein (Warren et al., 1975).

Acknowledgments

We thank J. Koudelka for preliminary work on this project.

References

Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W., & Fu, P. C. (1974) *Clin. Chem. (Winston-Salem, N.C.) 20*, 470.

Assmann, G., Sokoloski, E. A., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 549.

Avila, E. M., Hamilton, J. A., Harmony, J. A. K., Allerhand, A., & Cordes, E. H. (1978) *J. Biol. Chem. 253*, 3983.

Barenholz, Y., Gibbs, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.

Chen, G. C., Krieger, M., Kane, J. P., Wu, C. C., Brown, M. S., & Goldstein, J. L. (1980) *Biochemistry 19*, 4707.

Deckelbaum, R. J., Shipley, G. G., & Small, D. M. (1977) *J. Biol. Chem. 252*, 744.

Demel, R. A., Bruckdorfer, K. R., & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 255*, 321.
Eletr, S., & Inesi, G. (1972) *Biochim. Biophys. Acta 282*, 174.
Forte, T., & Nichols, A. V. (1972) *Adv. Lipid Res. 10*, 1.
Fredrickson, D. S., & Levy, R. I. (1972) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., & Fredrickson, D. S., Eds.) p 545, McGraw-Hill, New York.
Gotto, A. M., Levy, R. I., Lux, S. E., Birnhaumer, M. E., & Fredrickson, D. S. (1973) *Biochem. J. 133*, 369.
Griffith, O. H., & Jost, P. C. (1976) in *Spin Labelling: Theory and Applications* (Berliner, L. J., Ed.) p 453, Academic Press, New York.
Henkens, R. W., & Sturtevant, J. M. (1972) *Biochemistry 11*, 206.
Ho, Y. K., Brown, M. S., & Goldstein, J. L. (1980) *J. Lipid Res. 21*, 391.
Huang, C. (1969) *Biochemistry 8*, 344.
Lowry, O. H., Roseborough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Miller, G. J., & Miller, N. E. (1975) *Lancet 1*, 16.
Morrisett, J. P., Jackson, R. L., & Gotto, A. M., Jr. (1975) *Annu. Rev. Biochem. 44*, 183.
Newman, G. C., & Huang, C. (1975) *Biochemistry 14*, 3363.
Rhoads, G. G., Gulbrandsen, C. L., & Kagan, A. (1976) *N. Engl. J. Med. 294*, 293.
Rojers, J., Lee, A. G., & Wilton, D. C. (1979) *Biochim. Biophys. Acta 552*, 23.
Ruyle, W. V., Jacob, T. A., Chemerda, J. M., Chamberlin, E. M., Rosenburg, D. W., Sita, G. E., Erickson, R. L., Aliminosa, L. M., & Tishler, M. (1953) *J. Am. Chem. Soc. 76*, 2604.
Shen, B. W., Scanu, A. M., & Ke'zdy, F. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 837.
Small, D. M. (1970) in *Surface Chemistry of Biological Systems* (Blank, M., Ed.) Plenum Press, New York.
Smith, E. B. (1974) *Adv. Lipid Res. 12*, 1.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113.
Tall, A. R., & Lange, Y. (1978) *Biochem. Biophys. Res. Commun. 80*, 206.
Tardieu, A., Mateu, L., Sardet, C., Weiss, B., Luzzati, V., Aggerbeck, L., & Scanu, A. M., Jr. (1976) *J. Mol. Biol. 101*, 129.
Taylor, M. G., & Smith, I. C. P. (1980) *Biochim. Biophys. Acta 599*, 140.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622.
Warren, G. B., Houslay, M. D., Metcalfe, J. C., & Birdsall, N. J. M. (1975) *Nature (London) 255*, 684.
Yeagle, P. L. (1981) *Biochim. Biophys. Acta 640*, 263.
Yeagle, P. L., Langdon, R. G., & Martin, R. B. (1977a) *Biochemistry 16*, 3487.
Yeagle, P. L., Martin, R. B., Lala, A. K., Lir, H., & Bloch, K. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4924.
Yeagle, P. L., Martin, R. B., Pottenger, L., & Langdon, R. G. (1978) *Biochemistry 17*, 2707.
Zurcher, A., Heusser, H., Jeger, O., & Geistlich, P. (1954) *Helv. Chim. Acta 37*, 1564.

Rat Mast Cell Phospholipase A_2: Activity toward Exogenous Phosphatidylserine and Inhibition by *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylserine†

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ABSTRACT: The presence of phospholipase A_2 in intact rat peritoneal mast cells was investigated by using two synthetic radiolabeled phosphatidylserine (PS) substrates. Incubation of intact cells with 1-oleoyl-2-[^{3}H]oleoyl-PS resulted in the release of a considerable quantity of [^{3}H]oleic acid from the substrate. To establish that [^{3}H]oleic acid release was mediated via direct enzymatic attack at the *sn*-2 position, we measured release of the [^{3}H]serine moiety from the glycerol backbone of 1,2-dimyristoylphosphatidyl[^{3}H]serine. This activity, which represents the combined actions of phospholipases C and D, was 10-fold lower than [^{3}H]oleic acid release, indicating that neither of these enzymes is required for the release of the preponderance of [^{3}H]oleic acid. These results establish the existence in intact rat mast cells of a phospholipase A_2 active toward exogenous PS. Over the concentration range at which exogenous PS activates mast cell secretion, intact mast cells and broken cells possessed nearly equal levels of phospholipase A_2 activity, and enzyme activity was 3–4-fold higher toward PS than phosphatidylcholine. Several agents were tested for their ability to inhibit phospholipase A_2 in intact mast cells. Of the agents tested, an N-substituted derivative of PS previously identified as an inhibitor of mast cell secretion was shown to be a particularly potent and efficacious inhibitor of mast cell phospholipase A_2. The concentration dependence of enzyme inhibition paralleled inhibition of histamine secretion, providing a strong positive correlation between the level of phospholipase A_2 in mast cells and the capacity for secretion.

Of the many agents known to induce rat mast cells to secrete histamine, a subset is distinguished by dependence on exogenous phosphatidylserine (PS)[1] for a maximal response (Goth et al., 1971; Baxter & Adamik, 1978). Included in this group

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[1] Abbreviations: Con A, concanavalin A; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; NBD-PS, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylserine; PC, phosphatidylcholine; PS, phosphatidylserine.

0006-2960/82/0421-1254$01.25/0