Chiral Surfaces in Micelles of Enantiomeric *N*-Palmitoyl- and *N*-Stearoylserine

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Abstract: Circular dichroism (CD) spectra were recorded with micellar aggregates of a series of *N*-palmitoyl and *N*-stearoyl derivatives of amino acid enantiomers. *N*-Palmitoyl- and *N*-stearoyl-L- (or -D-) serine in the micelle form (10^{-4} M in aqueous 0.01 M KOH) exhibited a strong CD band centered at 213–215 nm which could be completely abolished by disintegrating the micelles in 50% ethanol. Analogous CD spectra of enantiomeric *N*-palmitoyl derivatives of tyrosine or proline did not display any exclusive band for the micellar form. The CD spectra of the enantiomeric *N*-palmitoyl- or *N*-stearoylserine micelles presumably originated from a repetitive arrangement of the amide planes on the micellar surface. Computer modeling suggested an alternating tilt of the amide planes associated with the formation of parallel spines of –NH···OC– intermolecular hydrogen bonds which cover the micellar surface. Each of such spines has a supramolecular chirality, which is presumably the origin of the observed CD band. The network of such chiral spines forms a unique chiral surface which may bear important implications for surface recognition and catalysis.

The adhesion forces in a condensed phase of chiral molecules are affected by a unique vectorial restriction which corresponds to the bulk asymmetry of the system. In an isotropic fluid of chiral molecules, this "chiral discrimination" may affect the dominant adhesive hydrogen bonds and van der Waals forces to an extent which may be reflected in small differences in properties such as boiling point, specific volume, and refractive index, with respect to the analogous racemic fluid.[1–4] In addition, optical rotation of chiral molecules in the liquid phase has a component of chiral discriminating intermolecular association which is abolished by dilution.[5]

Chiral discrimination may be greatly enhanced in a 2-dimensional condensed phase, as has been extensively investigated by Arnett and co-workers with monolayers of palmitoyl and stearoyl derivatives of chiral molecules,[6–9] in particular enantiomeric *N*-stearoylserine methyl esters.[7] These molecules form a distinct monolayer at the air–water interface, where the long hydrocarbon chains align parallel to each other at the air phase while the chiral head groups are closely packed to form a condensed phase. The chiral discriminating recognition in this special organization could be clearly demonstrated and characterized by surface pressure/area (Π/A) diagrams and other monolayer properties.[7–9]

The chiral molecular interactions in monolayers presumably form a network of repetitive fragments which may be regarded as a supramolecular chiral surface of a monomolecular thickness. In this study, we have simulated the monolayer surface of *N*-stearoylserine methyl ester[7] in anionic micelles made of *N*-acyl (-L- or -D-) serine in aqueous basic solution. The CD spectra of these micelles clearly indicated the formation of a unique supramolecular chiral organization on the micellar surface.

Experimental Section

All chemicals used were of the highest purity available. Solvents were either spectrograde or freshly double-distilled. Amino acids of high purity (>99%) were obtained from Fluka and used for synthesis as such.

***N*-Acyl Amino Acids.** *N*-Stearoylserine enantiomers were prepared either by reacting the *N*-hydroxysuccinimide ester of stearic acid with L- [*S*-(+)-] or D- [*R*-(–)-] serine (series I) or by hydrolysis of the *N*-stearoylserine methyl esters (series II), as outlined below. Stearic acid *N*-hydroxysuccinimide ester (SHE) was prepared by reacting 1 equiv of stearic acid (99.9% pure, Aldrich) with 1 equiv of *N*-hydroxysuccinimide (99%, Fluka) in ethyl acetate in the presence of 1 equiv of dicyclohexylcarbodiimide (from BDH) as previously described.[10] The product was crystallized once from ethyl acetate–petroleum ether and then from ethanol. The crystals melted sharply at 93 °C as reported[10] and displayed a single spot on a thin-layer chromatogram (R_f = 0.67, silica gel 60, Merck, running solvent chloroform–methanol–water (65:25:4)). SHE was reacted with L- or D-serine to yield the respective *N*-stearoylserine by a slight modification of a procedure described for *N*-lauroylserine.[10] A solution of 0.5 g of L- or D-serine in 50 mL of 0.1 M sodium carbonate–sodium bicarbonate was added with mixing to 0.5 g of SHE in 50 mL of tetrahydrofuran. The slightly opaque solution was allowed to mix for 18 h at 35 °C and then evaporated down to 50 mL and acidified with 3 N HCl to pH 1, whereupon the product precipitated, was collected by filtration, and was washed extensively with water. The product was crystallized once from acetone and twice from ethanol–water (4:1), yielding fine crystals melting at 103 °C (Fisher-Johns).[11] Both *N*-stearoyl-L- and -D-serine displayed a single spot on thin-layer chromatograms (R_f = 0.35 silica gel, chloroform–methanol–water (65:5:4)).

In series II, *N*-stearoyl-L- and -D-serine were prepared by hydrolysis of the respective methyl esters, which were kindly donated by Dr. A. M. Arnett, Duke University. The synthesis, purification, and physical properties of these esters were described in detail elsewhere.[7] A 0.1 M solution of *N*-stearoyl-L- or -D-serine methyl ester in methanol was mixed 1:1 (v/v) with 0.2 M KOH in water. The rate of hydrolysis was monitored by the liberation of *N*-stearoylserine determined by TLC (see above). After 18 h, the ester was completely hydrolyzed and yielded a single spot on a thin-layer chromatogram identical to that of the compounds of series I. The solution was then immediately used for spectral measurements.

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N-Palmitoyl-L-serine (mp 97–98 °C) and *N*-palmitoyl-D-serine (mp 97–98 °C) were prepared by reacting palmitic acid *N*-hydroxysuccinimide ester (Sigma, 99%, twice recrystallized from ethyl acetate–petroleum ether) with L-serine and D-serine as described above. Crystallization and assessment of purity were carried out as for the *N*-stearoyl analogues.

N-Acetyl-L-serine and *N*-acetyl-D-serine were prepared by reacting L- and D-serine with acetic anhydride as previously described[12,13] and purified on a Dowex 50 (H^+ form) column.[13]

N-Palmitoyl-L-tyrosine (mp 132 °C), *N*-palmitoyl-D-tyrosine (mp 133 °C), *N*-palmitoyl-L-proline (mp 69 °C), and *N*-palmitoyl-D-proline (mp 69 °C) were prepared and crystallized analogously to *N*-palmitoylserine. All four compounds were chromatographically pure.

Critical Micellar Concentration (cmc). Determination of cmc was performed by scattering of polarized light[14] measured with a fluorescence polarization instrument.[15] The intensity of a vertically polarized 546-nm-beam Hg band scattered by the sample was measured at a right angle through a vertically polarized analyzer free of filter.

Circular Dichroism (CD). CD spectra were recorded at 25 °C with a Jasco J-500C spectropolarimeter equipped with a DP-500N data processor, using 1 cm light path cells. Most recorded samples were of 10^{-4} M concentration, which was adjusted by optical density measurements at 205 nm. Each CD spectral scanning was repeated four times to ensure reproducibility, and in some cases spectral recordings were repeated with different samples.

Lipid Fluidity. This was determined by the steady-state fluorescence depolarization method[16] with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe.[15,16] The instrumentation and methodology are described in detail elsewhere.[15,16]

Results

A series of *N*-palmitoyl and *N*-stearoyl derivatives of L and D amino acids were first synthesized and then screened for their solubility in water at basic pH and the formation of stable transparent micellar suspensions. The most satisfactory were the serine, tyrosine, and proline derivatives, which formed relatively clear micellar solutions at concentrations around 10^{-4} M (see below) and were therefore selected for this study. The micelles of the long-chain amino acid derivatives were analyzed for chiral discriminative organization by CD spectroscopy in the range 200–260 nm, which corresponded primarily to the Cotton effect of the amide bond and could indicate repetitive chiral assemblies of the amide planes on the micellar surface.

***N*-Stearoyl- and *N*-Palmitoylserine.** All experiments with these compounds were carried out in 0.01 M KOH solution to ensure complete ionization. A 0.1 M stock solution of the tested serine derivative in ethanol was diluted 1:1000 with vortexing into aqueous 0.01 M KOH. Measurements were performed at 25 °C between 5 and 15 min after solution preparation.

The cmc values of the *N*-stearoylserine micelles were slightly lower for the chiral micelles than for the racemic micelles (2×10^{-5} and 4×10^{-5} M, respectively; see Figure 1). By definition, this difference stemmed from an additional chiral discriminative adhesive force in the chiral head group assembly, as has been previously noticed in monolayers of *N*-stearoylserine methyl esters.[7]

CD spectra were recorded in aqueous solutions above the cmc and in 1:1 water–ethanol solution, where the acylated serines are not aggregated. For all four enantiomeric serine derivatives, a drastic change in the CD spectrum accompanied the micellar state, as shown in Figures 2–5. Racemic micelles made by premixing equal amounts of the enantiomers practically abolished the CD spectra, as could be expected (see Figures 3 and 5). To account for the effect of ethanol on the spectra, we recorded the CD spectra of *N*-acetyl-L-serine (Figure 2) and *N*-acetyl-D-serine (not shown) under the same conditions. The CD spectra of *N*-acetyl-L-serine in aqueous solution and in 50% ethanol were similar in shape and magnitude to each other and to that of *N*-stearoyl-L-serine in 50% ethanol (Figure 2). CD spectra in 80% ethanol were virtually identical to those in 50% ethanol, pointing to the complete disintegration of the micelles in the latter. Micelles formed with *N*-stearoylserine of series II (see Experimental Section) yielded CD spectra essentially identical in both shape and magnitude to those presented for series I in Figures 2 and 3. The marked change in $\Delta\epsilon$ associated with micelle formation in the enantiomeric long-chain serine derivatives (Figures 2–5) predicts that a similar change in $\Delta\epsilon$ should take place upon increase in concentration. Figure 6 describes the concentration dependence of $\Delta\epsilon$ at 213 nm, the peak wavelength of the CD spectra (see Figures 2–5), of *N*-stearoyl-L- or -D-serine below and above the cmc. Here again, $\Delta\epsilon$ changes in both sign and magnitude upon micelle formation ($>2 \times 10^{-5}$ M). It is interesting to note that the absolute $\Delta\epsilon$ values at concentrations above the cmc keep increasing, which indicates that the chiral organization which is responsible for the change in $\Delta\epsilon$ has not reached its full capacity at 10^{-4} M.

Micelles of *N*-stearoylserine labeled with DPH displayed very high fluorescence polarization values similar to those obtained in liposomes of a tightly packed lipid domain (e.g. distearoyllecithin at room temperature).[16] At 25 °C, the recorded values of the fluroescence anisotropy (r) were in the range of 0.33, approaching the limiting value of $r_0 = 0.368$ for DPH,[15] which corresponds to a solid immobile phase. Under similar conditions, ordinary detergent micelles yield much lower fluorescence anisotropy values.[17] Figure 7 presents the temperature dependence of

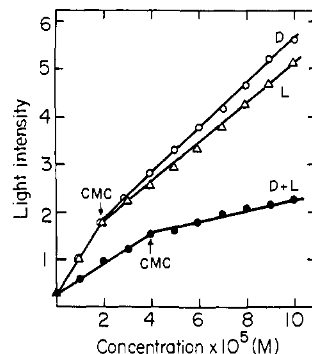

Figure 1. Determination of critical micellar concentrations (cmc's) of *N*-stearoyl-L-serine (△) *N*-stearoyl-D-serine (○), and a 1:1 mixture of them (●) in aqueous 0.01 M KOH. The figure presents the change in intensity of scattered vertically polarized monochromic light (546 nm, Hg band) with molar concentration of the serine derivative.

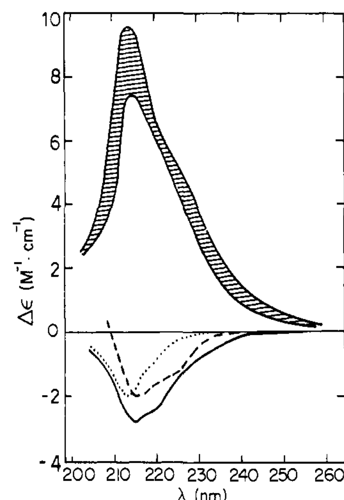

Figure 2. Upper panel: CD spectrum recorded with 10^{-4} M *N*-stearoyl-L-serine in aqueous 0.01 M KOH (micelles). The shaded area represents the range recorded with six different samples. Lower panel: CD spectra recorded with 10^{-4} M *N*-stearoyl-L-serine in 1:1 ethanol–water containing 0.01 M KOH (—) and with *N*-acetyl-L-serine in aqueous 0.01 M KOH (···) and in 1:1 ethanol–water containing 0.01 M KOH (- - -).

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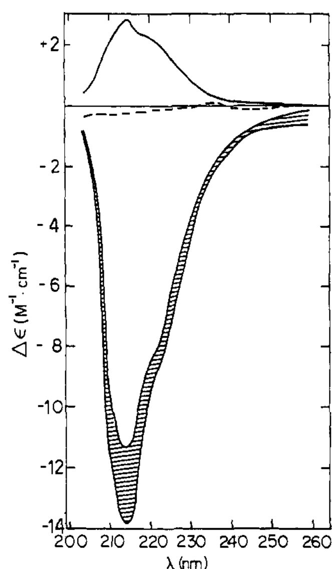

Figure 3. Lower panel: CD spectrum recorded with 10^{-4} M *N*-stearoyl-D-serine in aqueous 0.01 M KOH (micelles). The shaded area represents the range recorded with six different samples. Upper panel: CD spectrum recorded with 10^{-4} M *N*-stearoyl-D-serine in 1:1 ethanol–water containing 0.01 M KOH (—). The CD recording for a 1:1 mixture of *N*-stearoyl-D- and -L-serine in aqueous 0.01 M KOH is also presented (- - -).

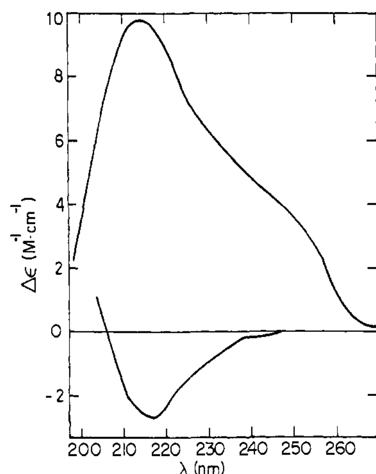

Figure 4. CD spectra recorded with 10^{-4} M *N*-palmitoyl-L-serine in aqueous 0.01 M KOH (upper curve) and in 1:1 ethanol–water containing 0.01 M KOH (lower curve).

r in enantiomeric and racemic micelles of *N*-stearoylserine. Since the scale of *r* is highly nonlinear,[16] the data in Figure 7 are replotted in Figure 8 in terms of $(r_0/r - 1)^{-1}$, which can be applied as a linear presentation of lipid microviscosity.[16] Deviation from linearity in a presentation of log $(r_0/r - 1)^{-1}$ *vs* $1/T$ can indicate phase changes or phase transitions.[16] The apparent inflections in Figures 7 and 8 could have stemmed from an experimental flaw or alternatively indicate pseudotransitions centered at 33 °C for the enantiomeric micelles and at 32 °C for the racemic micelles. Since the change in $(r_0/r - 1)^{-1}$ value below and above these temperatures is rather mild and remains of high absolute value, it may reflect a rearrangement of stretched stearoyl chains between two sets of dense packing. At 25 °C, where all CD spectra were recorded, the stearoyl chains in both the enantiomeric and racemic micelles were in a dense, highly ordered configuration.

N-Palmitoyltyrosine. The pK_a of the phenol residue in *N*-acyltyrosine[18] was assumed to be in the range 10–11. Therefore, the CD spectra of *N*-palmitoyl-L- and -D-tyrosine micelles were recorded in 0.1 M NaOH, where both the carboxyl and the phenol residues are essentially fully ionized, and at pH 8.4, where only the carboxyl residue was expected to stay fully ionized while the phenol residue remained un-ionized. The CD spectra of *N*-

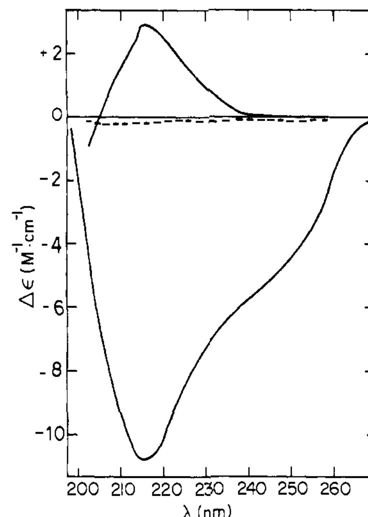

Figure 5. CD spectra recorded with 10^{-4} M *N*-palmitoyl-D-serine in aqueous 0.01 M KOH (lower curve) and in 1:1 ethanol–water containing 0.01 M KOH (upper curve). The CD recording for a 1:1 mixture of *N*-palmitoyl-L- and -D-serine is also presented (- - -).

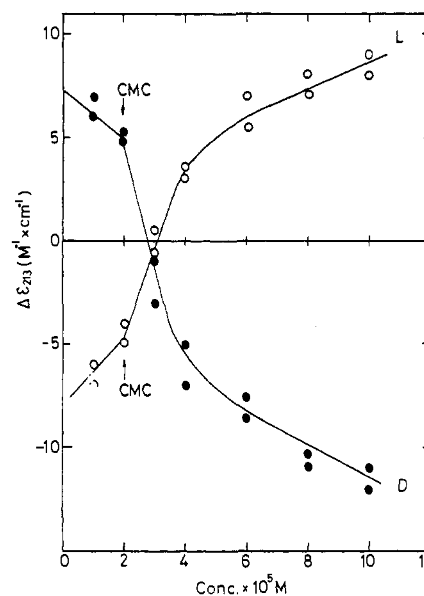

Figure 6. Change in Δε at 213 nm with concentration of *N*-stearoyl-L-serine (○) and *N*-stearoyl-D-serine (●) in aqueous 0.01 M KOH. Measurements were taken in 1- and 5-cm cells for concentrations above and below 4 × 10^{-5} M, respectively. Results of two independent experiments are presented.

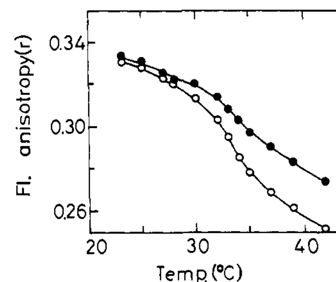

Figure 7. Change of fluorescence anisotropy (*r*) with temperature of DPH-labeled 10^{-4} M *N*-stearoyl-L-serine (●) and of a 10^{-4} M 1:1 mixture of *N*-stearoyl-L-serine and *N*-stearoyl-D-serine (○) in aqueous 0.01 M KOH.

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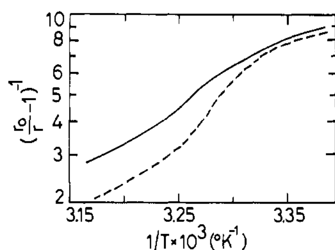

Figure 8. Replot of the data presented in Figure 6 as log $(r_0/r-1)^{-1}$ *vs* $1/T$.

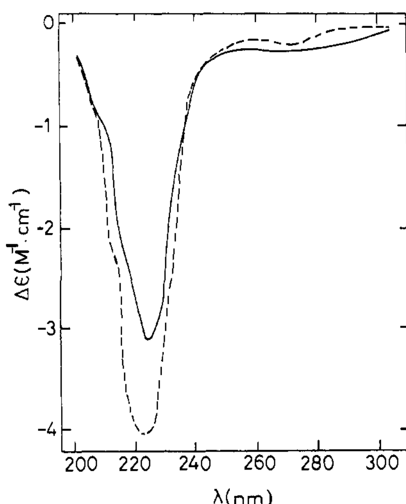

Figure 9. CD spectra recorded with 10^{-4} M *N*-palmitoyl-L-tyrosine in aqueous 0.01 M KOH (—) and in 1:1 ethanol–water containing 0.01 M KOH (- - -).

palmitoyl-L-tyrosine are displayed in Figure 9. The similarity between the CD spectra in water and in water–ethanol (1:1) indicates that the Cotton effect of the amide planes on the surface of these micelles is similar to that which appears when the molecules are not aggregated. The CD spectra above 240 nm were of low magnitude, which indicated that the Cotton effect of the phenol absorption is small and similar in magnitude in both the micellar and the dissociated forms. Analogous spectra of opposite signs were obtained for *N*-palmitoyl-D-tyrosine (not shown).

The CD spectra in 0.1 M NaOH were similar in shape and magnitude to those shown in Figure 9, which again do not provide indication for a possible chiral organization on the surface of *N*-palmitoyltyrosine micelles. Yet, the possibility that, in these micelles, chiral organizations, which do not imply periodic organization of the amide planes, nevertheless prevail[8] cannot be excluded.

***N*-Palmitoylproline.** The CD spectra of *N*-palmitoyl-L-proline in basic solutions are shown in Figure 10. In this compound, the alkylated amide bond lacks the ability to form a hydrogen bond with a neighboring amide, and therefore the difference in CD spectra displayed between the aqueous solution and the 50% ethanolic solution can be attributed primarily to solvent effects. Similar spectra of opposite signs were recorded for *N*-palmitoyl-D-proline (not shown).

Discussion

The CD spectrum of an N-acylated enantiomer of an amino acid is a superposition of two spectra. The first corresponds to the Cotton effect of the transition dipoles of the amide bond, and the second emerges from supramolecular chiral assembly of the amide planes, which can be effective only in a condensed phase. A specific chromophoric residue in the amino acid (e.g. the phenol side chain in L-tyrosine) may also contribute to the CD spectrum

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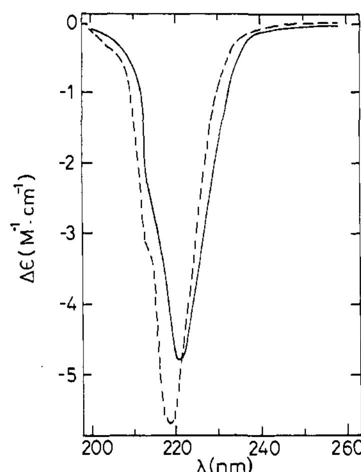

Figure 10. CD spectra recorded with 10^{-4} M *N*-palmitoyl-L-proline in aqueous 0.01 M KOH (—) and in 1:1 ethanol–water containing 0.01 M KOH (- - -).

in both ways. A convenient condensed phase for CD recording of chiral assemblies is the micelle. *N*-Palmitoyl or *N*-stearoyl amino acids form micelles in aqueous solution[14] with a cmc below 10^{-4} M (see for example Figure 1). Such micelles can be readily dissociated to monomers or small oligomers by the addition of an organic solvent, like ethanol, which provides a simple means for disintegration of chiral assemblies and assessment of their CD spectrum. In our study, only the micelles of the serine derivatives displayed a clear difference in CD spectra when they were recorded in aqueous solution or in 50% ethanol. These micelles were therefore studied in more detail.

Reproducible and meaningful CD spectra with *N*-palmitoyl- or *N*-stearoylserine could be recorded only at wavelengths above approximately 200 nm. In our recorded spectra, the CD peak at 213–215 nm could be attributed to the π–π^* transition of the amide bond while the shoulder at longer wavelengths presumably originates from the n–π^* transition.[19-21] The CD spectra of these transitions are extremely sensitive to coupling with neighboring amides, as in polyaminoacids, in particular when a chiral assembly is formed. Polyamino acids containing α-helix, β-sheet, or random-coil structures have each a typical CD spectrum which originates from a specific chiral organization of the amide planes.[22]

The recorded CD spectra of the studied *N*-acylserine derivatives in 50% ethanol (Figures 2–5) were all similar in shape, magnitude, and sign. In the ethanolic solution, these parameters were as could be expected for a true solution or for an unordered assembly of amide planes. In aqueous solution of *N*-palmitoyl- or *N*-stearoylserine, micelle formation is accompanied by a drastic change in all parameters of the CD spectrum (see Figures 2–5): the sign is reversed, the absolute magnitude increases by 4–6-fold, and the spectrum stretches to longer wavelengths. The CD spectra of the chiral micelles (Figures 2–5) are markedly different from those established for amide organization in α-helix, β-sheet, or random assembly.[22-24] Furthermore, the peak magnitude of $\Delta\epsilon$ at 213–215, which is even higher than that for the analogous peak of an α-helix of a polyamino acid,[22] decreases and the peak changes sign upon micelle disintegration by dilution (Figure 6). These unique spectral features indicate that, in the micellar assembly of the long-chain acylated L- or D-serine, the amide bonds form a network which has a distinct chiral feature. The association forces in this network presumably consist of hydrogen bonds which are supported and aligned by the strong hydrophobic

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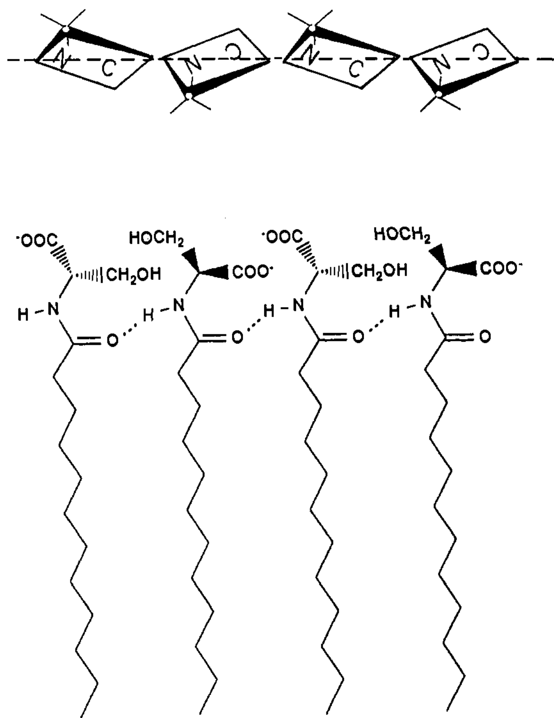

Figure 11. Side and upper views, of an assembly of four adjacent *N*-stearoyl-L-serine molecules in a micellar aggregate. The top view depicts the inter-amide chiral spine of the same segment including the asymmetric centers. A 2-dimensional criss-cross network of such spines will create a chiral surface.

interactions of the long acyl chains. It should be noted that light scattering may impose a suppressive effect on CD recordings, which is mostly pertinent to turbid or particulate suspensions.[25,26] However, in clear or slightly opaque solutions like micellar suspensions, the reduction in $\Delta\epsilon$ due to light scattering is not expected to exceed a few percent and for most considerations could be neglected.[27-29] The small deviations in $\Delta\epsilon$ magnitude observed with identical samples of *N*-stearoyl-L- or -D-serine displayed in Figures 2, 3, and 6 could be partially attributed to differences in light scattering due to changes in micellar size induced by microaggregation.

Our evaluation of the suggested supramolecular amide assembly in micelles of long-chain *N*-acyl derivatives of L- or D-serine was based on the following considerations: (1) The acyl chains in the micelles are tightly packed in an all-trans configuration, as indicated by the fluorescence polarization measurements (see Figures 7 and 8). Similar packing occurs in dipalmitoyl and distearoyl phospholipids below their phase transition.[16] (2) The serine residues and the amide bonds are all located at the micellar surface. (3) The carboxylate residues are at a maximal distance from each other due to charge repulsion. (4) The planes of the amide bonds are associated via intermolecular hydrogen bonds, –NH···O=C–, as in α-helix or β-sheet structures of polyamino acids. Computer analysis and modeling have suggested a specific assembly which complies with the above requisites. In this model, a network of parallel spines of amide planes covers the surface of the micelle. The spines are of intermolecular hydrogen bonds which propagate along the alternating amide planes. The directions of the serine head groups alternate 180° with alternating tilts in the amide planes, with respect to the hydrogen-bond spine. Figure 11 depicts a side view and a top view of a fragment of four molecules in such a spine. The spines are best packed parallel but displaced from each other by a half-molecular distance. Thus all second spines in this network appear as exact repetitions. In this model, the surface formed by the serine head group assembly has a unique chiral nature which has not yet been observed or suggested before. This chiral surface has obviously a mirror image and is probably the source of the characteristic CD spectra which were recorded here.

It is interesting to note the somewhat unexpected difference in the shape of the CD spectra between the *N*-palmitoylserine (Figures 4 and 5) and the *N*-stearoylserine micelles (Figures 2 and 3). It probably originates from a difference of tightness of the polyamide structures, which is induced by the hydrocarbon packing. Since CD spectra can be very sensitive to slight changes around the chiral centers, in reality the geometrical differences between the chiral surfaces of the enantiomeric *N*-palmitoyl- and the *N*-stearoylserine derivatives might be rather miniscule.

One peculiar observation which we have repeatedly noticed in the CD spectra relates to their magnitude. The absolute values of $\Delta\epsilon$ recorded for *N*-palmitoyl- or *N*-stearoyl-D-serine were always greater than those recorded for the analogous derivatives of L-serine. This could not be accounted for by partial racemization or the presence of impurities, since this difference could be practically abolished in ethanol–water (see Figures 2–5). Again, due to the high sensitivity of the CD spectra, this difference could be rather minute in structural terms but should nevertheless be addressed. The origin of this difference could be in principle related to the parity nonconserving energy difference between L and D enantiomers.[30] This minute energy difference may impose a marginal difference in the absolute $\Delta\epsilon$ value[31] which, however, is much smaller than the resolution power of any of the current instrumentation. Yet, one may propose that in the chiral aggregation a cooperative augmentation takes place which may amplify this difference by orders of magnitude into the detectable range. We have previously raised the speculation that a putative fundamental atomic property is inherent in asymmetric *R*- or *S*-carbons.[32] It remains degenerate at low concentrations but could emerge in condensed phases like micellar surfaces, where it could affect each chiral element slightly differently. It must be stressed, however, that the above possibilities are based on pure speculative arguments which remain to be investigated.

A class of amphipathic biological lipids which contain both an amide bond and a chiral center are the sphingolipids (e.g. sphingomyelin, cerebrosides, and gangliosides). The CD spectra of sphingolipid aggregates[33,34] display a band around 200 nm which is only moderately affected by alcohols.[33] Infrared spectra of sphingolipid aggregates[35] indicate hydrogen bonding of amide bonds, presumably between each other. Yet there is no CD indication for a possible chiral organization of the amide planes in sphingolipid micelles or liposomes.[33,34]

The proposed model for the chiral assemblage at the surface of the enantiomeric micelles of *N*-stearoyl- and *N*-palmitoylserine complies well with the surface organization at the air–water interface of monolayers of enantiomeric *N*-stearoylserine methyl esters, proposed by Arnett and his co-workers.[7] In both the monolayer and the micellar systems, chiral discrimination contributes to the increase, rather than decrease, in the adhesive forces.[7] It is therefore plausible that, in the air–water films of enantiomeric *N*-stearoylserine methyl esters,[7] the amide bonds are actually aligned in parallel spines similar to those proposed for the micelles (Figure 11). Such chiral surfaces may possess unique properties with far-reaching implications.

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