Smith, H. W. (1959), *J. Gen. Microbiol. 21*, 622.
Smith, L. D. S., and Holdeman, L. V. (1968), The Pathogenic Bacteria, New York, N. Y., Academic Press, pp 208, 219.
Stoffyn, P. J., and Jeanloz, R. W. (1954), *Arch. Biochem. Biophys. 52*, 373.
Svec, M. H., and McCoy, E. (1944), *J. Bacteriol. 48*, 31.
Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W (1963), *J. Amer. Chem. Soc. 85*, 2497.
Willis, A. T. (1969), Clostridia of Wound Infection, New York, N. Y., Academic Press, pp 51, 115.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Disruption of Low- and High-Density Human Plasma Lipoproteins and Phospholipid Dispersions by 1-Anilinonaphthalene-8-sulfonate*

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ABSTRACT: A hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS), at high concentrations was found to alter profoundly the structure of both low-density lipoproteins of the S_f 0–10 class and high-density lipoproteins of d 1.063–1.21. The disruption of these lipoproteins by 0.1 M NH_4ANS yielded essentially lipid-free proteins and phospholipid-rich and neutral lipid-rich fractions. Although all of the fractions obtained by the disruption of the lipoproteins were initially stabilized by association with ANS, the neutral lipid-rich fraction aggregated gradually upon loss of the ANS-stabilized phospholipid components. Lecithin dispersions were also disrupted extensively in 0.1 M ANS yielding an ANS–lecithin complex with an ANS:lecithin molar ratio of 2.4, a density of 1.16, and an approximate particle weight of 5.4×10^4. It appeared that ANS at high concentrations initially displaced the total lipid moiety from the lipoproteins.

The formation of phospholipid-rich and neutral lipid-rich fractions seemed to be effected by the progressive removal of the phospholipid-rich surface layer from the total lipid moiety.

Since Weber and Laurence (1954) introduced hydrophobic probes, 1-anilinonaphthalene-8-sulfonate (ANS)[1] and related organic compounds have been used extensively to study hydrophobic sites of proteins (Edelman and McClure, 1968; McClure and Edelman, 1966; Stryer, 1965; Brand *et al.*, 1967; Turner and Brand, 1968). These small organic anions possess a characteristic property of being practically nonfluorescent in water but highly fluorescent when dissolved in organic solvents or bound to hydrophobic sites of a number of proteins. In view of the hydrophobic character of the constituents of plasma lipoproteins, the study of the nature of the interaction between the lipoproteins and ANS was thought to be of considerable interest.

The present communication describes disruption by ANS of low-density lipoproteins of the S_f 0–10 class (LDL)[1] and high-density lipoproteins of d 1.063–1.21 (HDL) as well as phospholipid dispersions. High concentrations of ANS were found to release essentially lipid-free proteins from the lipoproteins.

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[1] Abbreviations used are: ANS, 1-anilinonaphthalene-8-sulfonate; LDL, low-density lipoproteins of the S_f 0–10 class; HDL, high-density lipoproteins of d 1.063–1.21.

Materials and Methods

ANS. The ammonium salt of ANS (K & K Laboratories, Inc., Jamaica, N. Y.) was purified by crystallizing three times from water as described for the magnesium salt of ANS (Weber and Young, 1964). The recrystallized ANS was further purified by column chromatography on silicic acid–Super Cel (2:1, w/w) employing chloroform–methanol (9:1, v/v) as the solvent. The solvent was evaporated using a Rinco evaporator and the purified ANS was stored over phosphorus pentoxide at 4° in a vacuum desiccator. During the purification, precautions were taken to minimize the exposure of ANS to laboratory lighting. Thin-layer chromatography of the purified ANS on silica gel G with chloroform–methanol–water (65:25:4, v/v) as the developing solvent indicated that the ANS preparation was essentially pure; a trace of slower moving contaminant became detectable only when the thin-layer plate was overloaded with sample.

Buffer. Unless otherwise indicated, experiments were performed with 0.02 M sodium phosphate buffer containing 0.1 M sodium chloride and 0.05% EDTA (pH 7.0).

Isolation of LDL and HDL. Recently outdated human blood containing 0.2 volume of citric acid–sodium citrate–dextrose anticoagulant solution was obtained from a local hospital as the source of plasma. For the separation of plasma lipoproteins pooled samples of the plasma free of turbidity were used. Plasma LDL of the S_f 0–10 class was isolated and purified by ultracentrifugation as previously described (Janado and Nishida, 1965). HDL ($1.063 < d < 1.210$) was isolated from the bottom fraction obtained by centrifugation of plasma

at *d* 1.063 (Bragdon *et al.*, 1956). The density of the fraction was increased to 1.21 by the addition of solid sodium bromide and the solution was centrifuged at 110,000*g* for 36 hr. The HDL was siphoned from the top of the tube and purified by recentrifugation at *d* 1.21 after a 1:4 dilution with a sodium chloride–sodium bromide solution of *d* 1.21. All centrifugations were carried out at 4° and the media used contained 0.025% EDTA. The LDL and HDL were dialyzed against phosphate buffer for 48 hr under nitrogen at 1° with four changes of the external solution and stored at 1° under nitrogen. Both LDL and HDL prepared in the manner described were shown to be free of each other and of albumin, the most likely contaminant, upon double diffusion by the method of Ouchterlony (1953); anti-human serum albumin used was obtained from Behring Diagnostics, Inc. (Woodbury, N. Y.) and anti-LDL and anti-HDL were produced in rabbits by the antigen injection method described previously (Chung and Nishida, 1967). Typical lipoprotein preparations used in this study contained 20.6% protein and 79.4% lipids for LDL and 48.5% protein and 51.5% lipids for HDL. The methods used for the determination are described in the following sections.

Lipoprotein Lipids. To obtain lipoprotein lipids, the extraction procedure of Folch *et al.* (1957) was modified. Aliquots of lipoprotein preparations were extracted with 25 volumes of chloroform–methanol (2:1, v/v) containing 0.2 ml of glacial acetic acid/100 ml. Acetic acid was included in the extraction mixture to facilitate precipitation of the proteins. The mixtures were heated at 55° under nitrogen for 15 min, shaken vigorously for 30 min, and centrifuged to remove the protein precipitate. The supernatant solution and chloroform–methanol washings were combined and mixed with 0.2 volume of water. The lower phase obtained after centrifugation of the mixture was washed 4 times with chloroform–methanol–water (3:48:47, v/v) and evaporated to dryness; the residues were dissolved in a known volume of chloroform. For the gravimetric determination of the total lipids, the chloroform solution was evaporated in a vacuum oven at 50° until a constant weight was obtained.

Protein Determination. Protein contents of LDL and HDL were routinely determined by the procedure described by Lowry *et al.* (1951) with crystalline bovine plasma albumin (Armour and Co., Chicago, Ill.) dried over phosphorus pentoxide as a standard. Lowry's color yield of total protein in intact LDL and HDL as compared to the albumin was 107 and 94%, respectively. These values were obtained from Kjeldahl nitrogen values (Minari and Zilversmit, 1963) determined for the proteins precipitated by the extraction of LDL and HDL and from the amino acid compositions (Levy *et al.*, 1967). Although Margolis and Langdon (1966) reported Lowry's color yield as 130% for LDL, our value was in agreement with the value, 110%, reported more recently by Levy *et al.* (1967).

Egg Lecithin. Crude egg lecithin was obtained from fresh egg yolks by the method described by Rhodes and Lea (1957) and purified by silicic acid column chromatography as described by Hanahan *et al.* (1957). Thin-layer chromatography of the purified lecithin on a silica gel G plate with chloroform–methanol–water (65:25:4, v/v) as the developing solvent gave a single spot of lecithin.

Phospholipid Dispersions. Aliquots of lipids in chloroform were placed in 25 × 65 mm tubes. After the solvent had been evaporated under nitrogen, 10 ml of buffer was added to the residue. The lipids were then sonified at 25° for 60 min using a Branson sonifier (Model S-75, 20 kc/sec, Branson Instruments, Inc., Danbury, Conn.) equipped with a step horn at position 7 of the power setting; the tuning dial was adjusted to give the maximum power supply meter reading. The temperature was monitored with a thermocouple. A methanol–water–Dry Ice bath was employed for cooling, and nitrogen gas was flushed over the surface during the sonification. Large aggregates were removed by centrifugation at 64,400*g* for 30 min.

Determination of Lipids. Lipid phosphorus was determined essentially according to the method of Bartlett (1959) as modified by Parker and Peterson (1965). The amount of phospholipid in the samples is given as the amount of phosphorus ×25.

For the determination of total cholesterol, samples were saponified and the cholesterol extracted according to the procedure described by Abell *et al.* (1952). The reagent described by Zak *et al.* (1957) was used for color development of the cholesterol with cholesterol purified by the bromination–debromination procedure (Schwenk and Werthessen, 1952) as a standard. Free cholesterol was precipitated as the digitonide which was then washed as described by Zak *et al.* (1957); the color was developed in the same manner as for total cholesterol. To calculate the amount of cholesterol ester in lipoprotein lipids, the difference in the total and free cholesterol contents was multiplied by 1.71.

The triglyceride content was determined as described by Zilversmit (1965) for neutral lipid fractions obtained by silicic acid column chromatography of total lipids as described by Van Handel and Zilversmit (1957).

The results of all lipid analyses were given as the means of three or four separate determinations. Standard deviation of the separate determinations did not exceed 6% and was usually 1–4%.

Density Gradient Centrifugation. Sucrose density gradients were prepared by layering eight 0.5-ml portions of buffer–sucrose solutions of decreasing density and 1 ml of buffer. The solutions layered contained 0.01 or 0.1 M NH_4ANS, or no ANS. All solutions were deaerated with nitrogen immediately before preparation of the gradients. Lipoprotein samples were placed as the top three layers on gradients having corresponding ANS concentrations. Control gradients were prepared in a similar manner. In a later phase of study, a Büchler density gradient mixer (Büchler Instruments, Inc., Fort Lee, N. J.) was used for preparation of the sucrose density gradients; 0.5 ml of lipoprotein samples was layered on the top of the gradients. Samples were centrifuged at 114,700*g* for 48 hr at 17° in a Spinco SW-39 swinging-bucket rotor. After centrifugation, 0.3-ml fractions were collected by puncturing the bottom of the tube with a Büchler piercing unit. Refractive indices were measured at 20° with a Zeiss refractometer and the densities were obtained from standard curves constructed for the gradient solutions of known densities.

Treatments of Sucrose Density Gradient Fractions for Chemical Analysis. Since ANS interfered with Lowry's protein determination, the following procedure was developed to permit estimation of LDL and HDL protein. Aliquots of the density gradient fractions were diluted 1:1 with water, and 25 volumes of chloroform–methanol (2:1, v/v) containing 0.2 ml of glacial acetic acid/100 ml was blown directly into the sample and mixed to give a fine dispersion of the protein and sucrose. ANS was solubilized in the extraction solvent together with lipoprotein lipids which were then freed of ANS by the subsequent washings described in the extraction procedure for the separation of lipoprotein lipids. For the protein determination, the precipitate obtained after removal of both lipids and ANS was washed twice with 2-ml portions of 1% phosphotungstic

acid in 0.4 N HCl to remove sucrose and any entrapped ANS. After centrifugation, the phosphotungstic acid solution was carefully decanted and the tubes were allowed to drain prior to protein assay. The protein precipitates were dissolved by adding 0.25 ml of 2 M NaOH and then warming the samples in a water bath for 20 min at 50°. The contents were mixed occasionally during the heating period. After cooling, 0.25 ml of water was added and the protein was assayed by the method of Lowry *et al.* (1951) as previously described. Lowry's color yield obtained by this procedure were 14.5% and 10.5% lower for LDL and HDL, respectively, than when the lipoproteins were assayed directly. Complete removal of ANS with the procedure described was indicated by the same color yield obtained with control lipoproteins and the lipoprotein samples containing 0.1 M ANS and sucrose.

Spectrophotometric Determinations. All spectrophotometric determinations were conducted with a Cary Model 11M spectrophotometer. Measurements were made using quartz cells with a 10-mm light path. When the volumes of solutions to be analyzed were less than 3-ml, quartz microcells were used.

Equilibrium Dialysis. Cellulose dialysis membranes (Union Carbide, Chicago, Ill.) were soaked in distilled water overnight, rinsed, and placed in gently boiling distilled water for 1 hr. The water was poured off and the membranes were rinsed several times with distilled water. The washing procedure was repeated with 1% sodium bicarbonate and twice with distilled water. The washed membranes were stored at 4° in distilled water. An acrylic plastic multicavity dialysis cell (Chemical Rubber Co., Cleveland, Ohio) having a 1-ml volume/half-dialysis chamber was used for equilibrium dialysis. A multipurpose rotator (Scientific Industries, Inc., Springfield, Mass.) was employed to provide agitation. To minimize oxidation of the samples, the dialysis chambers and all solutions prepared in phosphate buffer were flushed with nitrogen. Dialyses were performed for 20 hr in the dark at 5° unless otherwise noted. At all concentrations of ANS used, longer periods of dialysis did not change the distribution of ANS in the two chambers.

After dialysis, free ANS concentrations were determined for the solutions from the buffer side of the cell by measuring the optical densities at 350 and 266 mμ (Stryer, 1965) after proper dilution with buffer. The number of moles of bound ANS per mole of lecithin or per milligram of lipoprotein (r) was determined as described by Rosenberg and Klotz (1960) using the following relationship: $r = (D_t - D_f - D_m)/P$, where D_t is the number of moles of dye present; D_f, the number of moles of free dye; D_m, the number of moles of dye bound to the membrane determined directly in the absence of lecithin or lipoproteins as a function of D_f; and P, the amount of lipoprotein or lecithin present. At ANS concentrations used for both lipoproteins and lecithin dispersions, the binding process appeared to be reversible; the value of r was not influenced by incorporation of ANS in either the buffer side or sample side of the dialysis cells.

Analytical Ultracentrifugation. Ultracentrifugal analyses were performed with a Beckman-Spinco Model E ultracentrifuge. A standard double-sector cell and a synthetic boundary double-sector cell were utilized for sedimentation velocity measurements which were conducted at 52,640 rpm, 20°, bar angle of 55 deg. Synthetic boundaries were formed as described by Richards *et al.* (1968); 0.44 ml of solvent was placed in the left sector and 0.14 ml of sample was placed in the right sector. The schlieren optical system was used for determination of sedimentation coefficients and diffusion coefficients and for the molecular weight determination by Archibald's approach to sedimentation equilibrium. For these determinations the methods outlined by Schachman (1957) were used. Sedimentation coefficients are reported either as the observed value (s_{obsd}) or as $s_{20,w}$ and diffusion coefficients are reported as $D_{20,w}$. Corrections were made as described by Schachman (1957). Density measurements were made using a calibrated 5-ml pycnometer at 20° and viscosity was measured in a capillary Ostwald viscometer having a flow time of 297 sec for water at 20°. The apparent specific volume, V_{app}, was obtained from the equation

$$V_{app} = \frac{1}{d_0} - \frac{1}{x}\frac{d - d_0}{d_0}$$

where d is the density of solution, d_0 is the density of solvent, and x is the concentration of lipoprotein or other substance in grams per ml. As a partial specific volume of LDL, the value, 0.967 (Janado and Nishida, 1967), which is in close agreement with the values recently reported by Mauldin and Fischer (1970) was used. The value for HDL preparation used, 0.88, was calculated from density data obtained with a 5-ml pycnometer. Apparent specific volumes of lecithin–ANS and lipoprotein–ANS complexes were calculated from apparent specific volume of each component assuming the additivity of molar volumes. Apparent specific volumes of lecithin and ANS were determined to be 0.972 and 0.730, respectively. The molecular weight determination by the sedimentation equilibrium method was performed with Rayleigh interference optics according to the method described by Richards *et al.* (1968). Further information on the molecular weight determination is given in the text. All measurements were made on a Nikon Model 6 shadowgraph. Areas under the peaks were measured with a planimeter on tracings of 10× (linear) enlargements of the photographic plates.

Results

The addition of ANS to LDL profoundly influenced the ultracentrifugal behavior of the lipoproteins. The increase in the sedimentation coefficient of the lipoproteins upon addition of ANS (Table I) was apparently caused by the association of ANS with the lipoproteins since at the ANS concentration of 3.43×10^{-3} M, 0.97 μmole of ANS was found to be in association with 5.9 mg of LDL by equilibrium dialysis. When ammonium ANS was added to LDL to a final concentration of 9.6×10^{-3} M, an increase in the sedimentation coefficient was accompanied by a decrease in the peak area of the parent LDL–ANS complex and by the appearance of fast-sedimenting peaks (Figure 1, picture B). An increase in the ANS concentration to 4.8×10^{-2} M drastically changed the ultracentrifugal pattern; in addition to small fast moving peaks, a large slow moving peak (s_{obsd} = 2.9 S) was observed (picture C). At ANS concentrations greater than 7.3×10^{-2} M, the slow moving peak (s_{obsd} = 2.4 S) was accompanied by a sharp fast moving peak (s_{obsd} = 15 S) and a small faster moving peak (s_{obsd} = 21 S) (picture D). A similar pattern was obtained with LDL in 0.1 M ANS; the apparent sedimentation coefficients of the slow moving peak and the major fast moving peak were 2.0 and 14.5 S, respectively. When the mixture was allowed to stand overnight at 4° under nitrogen, the slow moving component was in part converted into a floating peak (picture E) while the sedimentation behavior of the 14.5S component was unchanged. The sedimentation coefficient of HDL also increased upon addition of ANS (Table I). Unlike LDL, when HDL was mixed with ANS of high con-

TABLE I: Effect of ANS on the Sedimentation Coefficient of LDL and HDL.[a]

Lipoproteins	ANS (M)	Major Peak $s_{obsd} \times 10^{13}$ S	Major Peak $s_{20,w} \times 10^{13}$ S	Minor Peaks $s_{obsd} \times 10^{13}$ S	
LDL	0	6.2	7.9		
LDL	3.43×10^{-3}	8.6	10.3		
LDL	9.63×10^{-3}	10.5		14.4	
LDL	1.93×10^{-2}	11.9		16.9	
LDL	4.8×10^{-2}	2.9		15.6	22.1
LDL	7.3×10^{-2}	2.4		15.3	20.8
LDL	9.5×10^{-2}	2.0		14.5	20.5
HDL	0	4.6	5.0		
HDL	5.02×10^{-4}	4.9	5.3		
HDL	2.01×10^{-3}	5.2	5.6		
HDL	9.5×10^{-2}	2.5			

[a] Observed sedimentation coefficient (s_{obsd}) is given for all samples and $s_{20,w}$ is given only for the lipoproteins whose structures were not altered as judged from the sedimentation behaviors (Figures 1 and 2). All values are from single determinations of representative experiments. Mixtures contained 5.9 mg/ml of LDL or 2.9 mg/ml of HDL and the indicated amounts of ANS in 0.1 M sodium chloride–0.02 M phosphate buffer (pH 7.0) containing 0.05% EDTA.

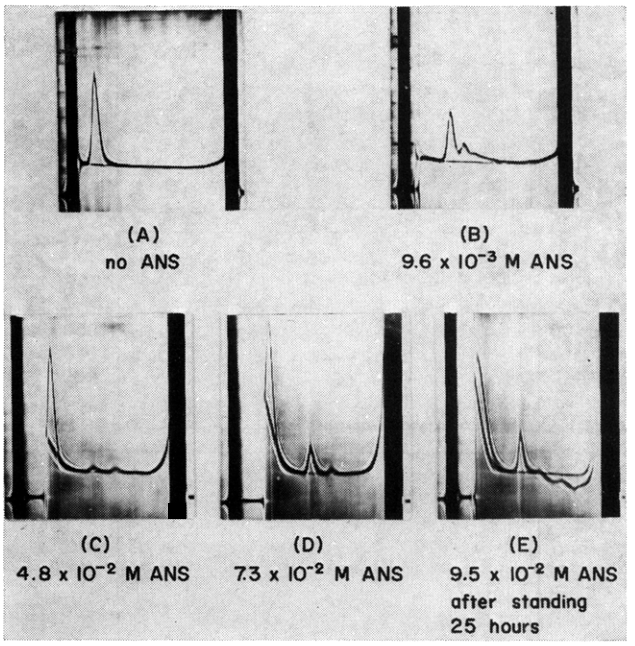

FIGURE 1: Effect of NH_4ANS on the ultracentrifugal behavior of LDL. Centrifugal patterns were obtained 24 min after the rotor reached a maximum speed of 52,640 rpm. Acceleration time, 5 min 20 sec. LDL concentration for all runs, 5.8–6.0 mg/ml. Medium, 0.1 M sodium chloride–0.02 M phosphate buffer–0.05% EDTA (pH 7.0).

centrations, no separation of boundaries was observed (Figure 2, picture B). However, when HDL in 9.5×10^{-2} M ammonium ANS was kept overnight at 4° or prepared in D_2O-buffer medium of d 1.104, floating peaks were produced (pictures C and D).

It was speculated that the appearance of the fast-sedimenting peaks at low ANS concentrations (Figure 1, picture B) represented a mere aggregation of the LDL–ANS complex. The pattern (picture C), at an ANS concentration of 5×10^{-2} M, may reflect the disruption of LDL into a slow moving lipid fraction and a protein fraction; the protein appeared only partially soluble at this concentration since further increases in the ANS concentration increased the size of the fast moving peaks (picture D). The floating peak (picture E) may have been caused by a release of neutral lipids from the total lipid fraction upon prolonged standing. Although HDL did not show the separation of lipid and protein boundaries, even at high ANS concentrations, the appearance of floating peaks (Figure 2, pictures C and D) seems to have been caused by the disruption of HDL. The lack of separation of the lipid and protein boundaries (picture B) was likely due to a similarity in the sedimentation coefficients of these components associated with ANS. These speculations were substantiated by results obtained with density gradient centrifugation of LDL and HDL in the presence of ANS.

In the absence of ANS, LDL and HDL samples gave essentially single peaks after centrifugation in sucrose density gradients at 114,700g for 48 hr at 17° (Figure 3A and Figure 4A). In gradients containing 0.01 M ANS, both LDL and HDL peaks moved further in comparison to control samples (Figure 3B and Figure 4B). The increase in the density of the lipoproteins by their association with ANS (d 1.370) seemed responsible for their greater migration in the sucrose gradient.

In contrast to the HDL–ANS band, the LDL–ANS band showed some turbidity. However, the appearance of a single LDL band supports the previous contention that in 0.01 M ANS, the LDL–ANS complex forms a series of aggregates (Figure 1, picture B).

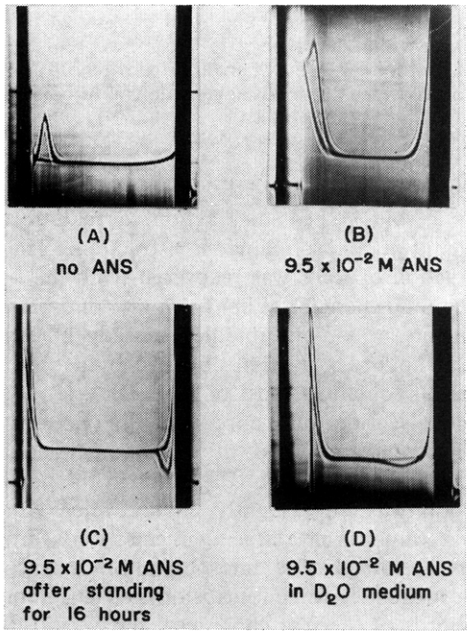

FIGURE 2: Effect of ANS on the ultracentrifugal behavior of HDL. Centrifugal patterns A, B, C, and D were obtained 24, 40, 8, and 16 min, respectively, after the rotor reached 52,640 rpm. HDL concentration, 2.9 mg/ml except for C, 5.9 mg/ml. Medium, 0.1 M sodium chloride–0.02 M phosphate buffer–0.05% EDTA (pH 7.0). D_2O medium used for D had the same electrolyte composition and the density of 1.104.

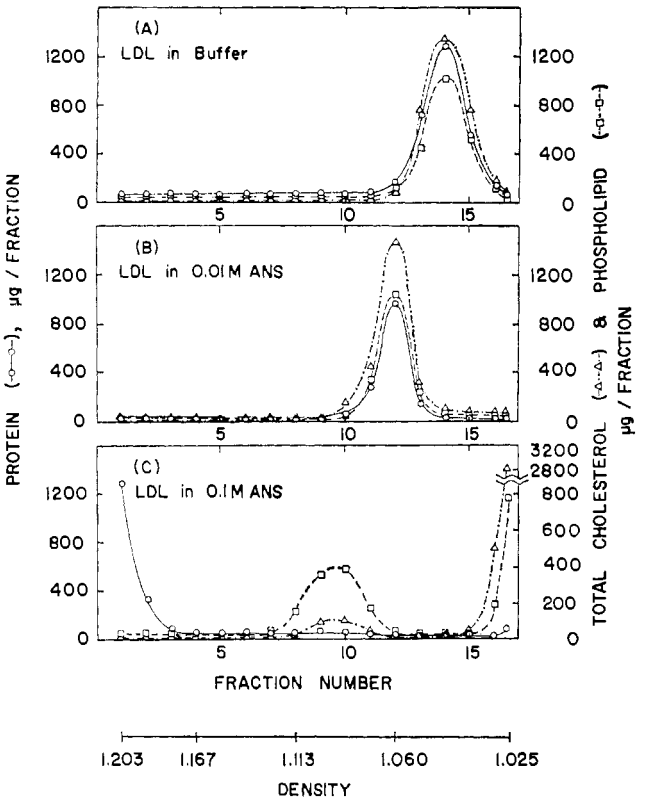

FIGURE 3: Density gradient centrifugation of LDL and LDL–ANS mixtures. LDL and LDL–ANS mixtures were centrifuged in sucrose density gradients for 48 hr at 114,700*g*. The samples for A, B, and C contained 11.5, 11.3, and 13.0 mg of LDL, respectively. After centrifugation, the distribution of protein, phospholipids, and total cholesterol was determined. Both the fraction number and density are given on the abscissa. The *d* 1.203 on the abscissa represents the density of the first fraction of the gradient from the bottom. The recovery of protein is given on the left ordinate and that of cholesterol and phospholipid on the right. All figures show the results obtained from single representative experiments. The total recoveries of protein, phospholipids, and total cholesterol from density gradients were within the range of 95–105% except for LDL protein from the 0.1 M ANS gradient where approximately 11% of the protein was not recovered due to the aggregation at the bottom.

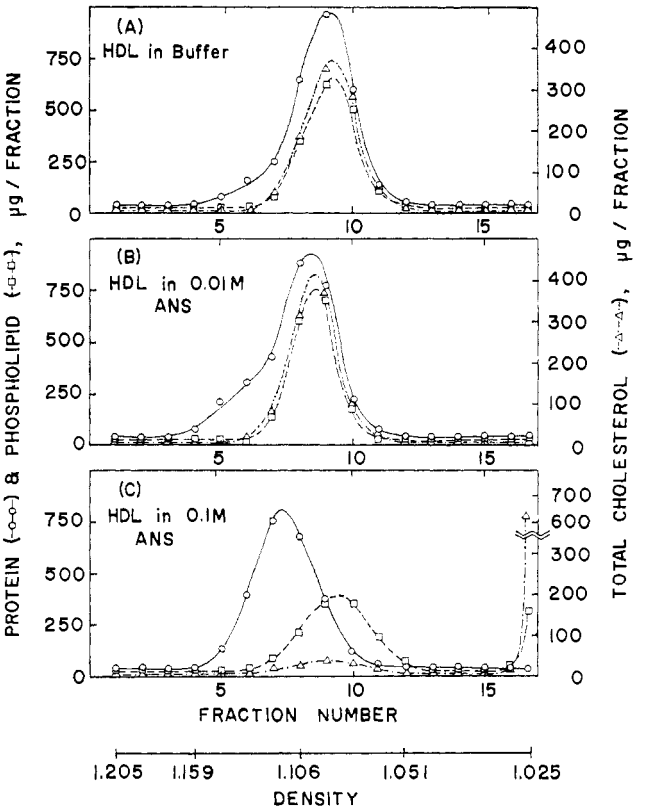

FIGURE 4: Density gradient centrifugation of HDL and HDL–ANS mixtures. HDL and HDL–ANS mixtures were subjected to density gradient centrifugation as described for Figure 3; the amount of HDL applied was 5.6 mg for A and B, and 5.0 mg for C. All figures represent results obtained from single experiments. The recoveries of protein, phospholipids, and total cholesterol from density gradients were within the range of 95–105% in all experiments.

In 0.1 M ANS, LDL and HDL were disrupted into three fractions: a soluble protein fraction, a neutral lipid-rich fraction, and a phospholipid-rich fraction. The soluble protein fraction of LDL was recovered from the bottom of the gradient and the neutral lipid-rich and phospholipid-rich fractions from the top and middle, respectively (Figure 3C). The neutral lipid-rich fraction obtained by density gradient centrifugation contained most of the LDL-esterified cholesterol and triglyceride; only approximately one-fourth of the phospholipid and one-third of the free cholesterol were found in this fraction (Table II). Although the neutral lipid-rich fraction was only slightly cloudy when collected, it gradually became turbid; large aggregates were formed after several days storage. The phospholipid-rich fraction contained approximately three-fourths of the LDL phospholipid and two-thirds of the free cholesterol but very little cholesterol ester and triglyceride. The lipid compositions of the phospholipid-rich and neutral lipid-rich fractions are also given (Table III). The protein fraction obtained was essentially lipid free; no measurable amount of cholesterol was present, and phospholipid and triglyceride contents were 0.7 and 2.2%, respectively (Table IV). Dialysis of this fraction against 0.1 M ANS and subsequent centrifugation gave major and minor sedimentation peaks (Figure 5) with apparent sedimentation coefficients of 14.6 and 19.3 S, respectively. These values corresponded to those of the fast-moving peaks obtained with LDL in 0.07 or 0.1 M ANS (Figure 1, picture D). Recently a sedimentation coefficient of 2.15 S was reported for succinylated LDL protein (Scanu *et al.*, 1968). Although the molecular weight of the LDL protein–ANS complex was not determined, the high sedimentation coefficients of the ANS-stabilized protein may be due largely to considerable intermolecular association of the protein subunits in view of the essentially same density (1.37) of apo-LDL (Scanu *et al.*, 1968) and ANS. The $s_{20,w}$ values computed for the major and minor ANS–protein peaks assuming the partial specific volume of 0.73 were 16.8 and 22.2 S, respectively. These values were not corrected for the Johnston–Ogston effect.

When HDL in 0.1 M ANS was subjected to sucrose density gradient ultracentrifugation for 48 hr at 114,700*g*, phospholipid-rich and neutral lipid-rich fractions were also formed (Figure 4C). In contrast to the results obtained with LDL, the HDL protein did not move to the bottom of the gradient, but appeared as a band which considerably overlapped that of the phospholipid-rich band. The recovery of HDL protein at density 1.11 appeared to be due to a smaller molecular weight than that of LDL protein and not due to the presence of lipids in the protein fraction. This was indicated by our observation that HDL protein obtained by extraction

TABLE II: Distribution of LDL and HDL Lipids between the Phospholipid-Rich and Neutral Lipid-Rich Fractions and Protein Fractions.[a]

Lipids	LDL			HDL		
	Phospholipid-Rich Fraction (%)	Neutral Lipid-Rich Fraction (%)	Protein Fraction (%)	Phospholipid-Rich Fraction (%)	Neutral Lipid-Rich Fraction (%)	Protein Fraction (%)
Phospholipid	75.3	24.5	0.2	85.0	13.7	1.3
Total cholesterol	18.9	81.1	Trace	25.5	74.5	Trace
Free cholesterol	64.0	36.0		71.7	28.3	
Cholesteryl ester	2.7	97.3		16.8	83.2	
Triglyceride	14.9	79.2	5.9	20.0	70.6	9.4

[a] Density gradient centrifugations of LDL and HDL in the presence of 0.1 M ANS were performed in the manner described for Figures 3 and 6, respectively. Pooled fractions were used for lipid analyses. The amounts of lipids associated with the protein fraction from HDL was computed by correcting the lipid contents of the lower portion of the protein band (fraction 5–7, Figure 6) for the protein recovery, assuming a homogeneous distribution of lipids in the entire protein fraction. The values in the table represent the means of three or four determinations.

TABLE III: Lipid Composition of the Phospholipid-Rich and Neutral Lipid-Rich Fractions of LDL and HDL.[a]

Lipids	LDL			HDL		
	Control (%)	Phospholipid-Rich Fraction (%)	Neutral Lipid-Rich Fraction (%)	Control (%)	Phospholipid-Rich Fraction (%)	Neutral Lipid-Rich Fraction (%)
Phospholipid	28.8	72.1	11.9	49.6	79.5	15.2
Free cholesterol	10.6	19.9	5.7	4.1	5.2	2.4
Cholesteryl ester	51.1	3.9	71.3	35.0	10.8	63.5
Triglyceride	9.5	4.1	11.1	11.3	4.5	18.9

[a] Lipid compositions of the two lipid fractions were obtained from the same lipid analysis given for Table II.

of HDL with $CHCl_3$–MeOH (2:1, v/v) at 4° and dissolved in 0.1 M ANS moved to the same position upon density gradient centrifugation in 0.1 M ANS. Furthermore, we obtained a recovery of HDL protein at a higher density (1.19) and also a greater separation of the phospholipid and protein bands by centrifugation for 72 hr at 124,000*g* in a sucrose density gradient prepared in 81 mole % D_2O (Figure 6). The protein recovered from the lower portion of the protein band (fraction 5–7) was almost free of lipids (Table IV) as was observed with LDL protein fraction. The residual lipids consisted of phospholipids and triglycerides, the contents being 0.2 and 1.2%, respectively. It has previously been reported that apo-HDL obtained by solvent

TABLE IV: Composition of the Protein Fractions Obtained by Density Gradient Centrifugation of LDL and HDL in the Presence of 0.1 M ANS.[a]

Constituents	LDL Protein Fraction (%)	HDL Protein Fraction (%)
Protein	97.6	98.1
Phospholipids	0.2	0.7
Cholesterol	Trace	Trace
Triglyceride	2.2	1.2

[a] The values were computed from the lipid analysis given for Table II and from the amount of proteins recovered from the protein fractions.

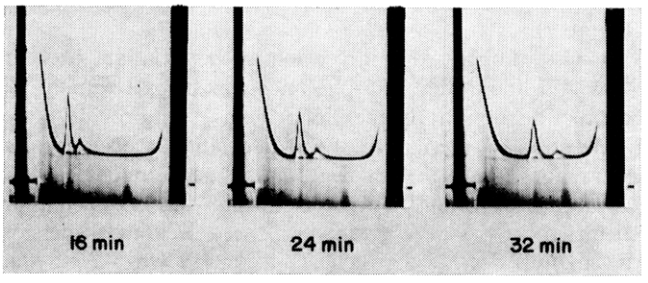

FIGURE 5: Ultracentrifugal patterns of LDL protein isolated by density gradient centrifugation of LDL in 0.1 M ANS. The density gradient centrifugation was performed in the manner described for Figure 3. The isolated protein was dialyzed against 0.1 M ANS in 0.1 M sodium chloride–0.02 M phosphate buffer–0.05% EDTA (pH 7.0). The centrifugal patterns obtained 16, 24, and 32 min after the rotor reached a maximum speed of 52,640 rpm are given. Acceleration time, 5 min 20 sec. Protein concentration, 2.25 mg/ml.

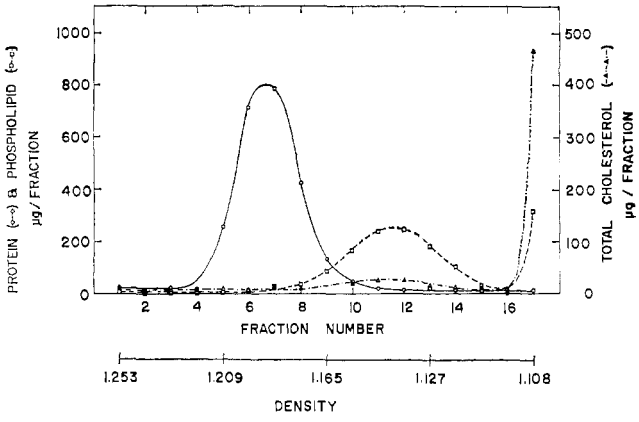

FIGURE 6: Density gradient centrifugation of HDL in the presence of 0.1 M ANS. Samples containing 5.0 mg of HDL in 0.1 M ANS were centrifuged for 72 hr at 124,000*g* in a sucrose density gradient prepared in 81 mole % D_2O–0.01 M ANS buffer using a Büchler density gradient mixer. The curves were obtained from a single experiment. The recoveries of protein, phospholipids, and total cholesterol from the gradient were 94, 97, and 96%, respectively.

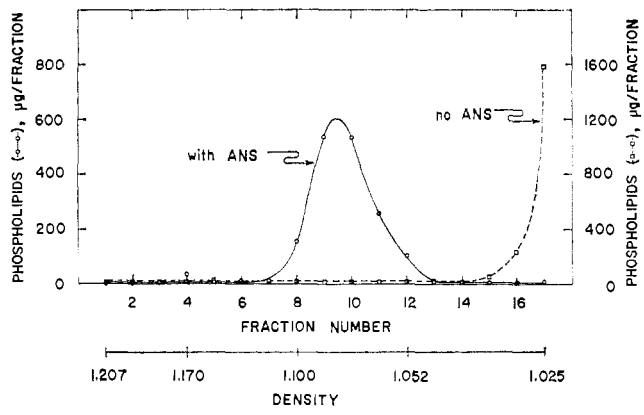

FIGURE 7: Density gradient centrifugation of LDL phospholipid dispersions in the presence and absence of 0.1 M ANS. The phospholipid dispersions (1 ml; 1.96 mg of phospholipid/ml) containing ANS or no ANS was applied to the top of the gradient and centrifuged in the manner described for Figure 3. The figure represents the results of single experiments. The total recoveries of phospholipid from the gradients were 96% in the absence of ANS and 87% in the presence of ANS. The LDL phospholipid was obtained by column chromatography of LDL total lipids; after elution of the neutral lipid with chloroform, the phospholipid was eluted with methanol.

extraction does not contain a significant amount of triglyceride (Scanu, 1966; Sodhi and Gould, 1967). Relatively high triglyceride contents in HDL protein fraction as well as LDL protein fraction may possibly reflect somewhat inefficient displacement of triglyceride by ANS in comparison to other lipid constituents. The distribution of the HDL lipids between the phospholipid-rich and neutral lipid-rich fractions was similar to that obtained with LDL (Table II). Phospholipids and free cholesterol were preferentially associated with the phospholipid-rich fraction while major portions of the cholesterol ester and triglyceride were recovered from the neutral lipid-rich fraction. The lipid composition of these fractions is given (Table III).

In the present study, density gradient centrifugation was employed to separate the products of lipoprotein disruption by virtue of their mobilities rather than to achieve isopycnic equilibrium with the gradient media. Nevertheless, the recovery of the phospholipid-rich fraction at densities considerably higher than those of their constituents (Figures 3C and 6) suggested that a large amount of ANS was complexed with the phospholipids. This speculation was substantiated by the results of sucrose density gradient centrifugation of LDL phospholipid dispersions in the presence and absence of ANS. In 0.1 M ANS, the phospholipid moved to the center of the gradient, while in the absence of ANS the phospholipid was recovered from the top of the gradient (Figure 7).

It was thought that an extensive association between ANS and lipoprotein phospholipids was in part responsible for the disruption of the lipoproteins. To provide some information on the nature of the association, we studied the interaction of ANS with egg lecithin as a model system. Ultracentrifugal analysis revealed that the sedimentation coefficient ($s_{20,w}$) of a freshly prepared lecithin dispersion (6.4 mg/ml) decreased from 4.71 to 2.44 S when ANS was added to final concentration of 0.1 M. The decrease in the sedimentation coefficient seems to have been caused by disruption of the phospholipid aggregates into much smaller units. If there had been no disruption, the association of ANS with the phospholipid would be expected to increase the phospholipid particle weight and density and hence the sedimentation velocity.

To determine the extent of association of ANS with egg lecithin at ANS concentration of 0.1 M, 0.5 ml of an egg lecithin dispersion (12.8 mg/ml) in 0.1 M ANS was dialyzed against 0.5 ml of 0.1 M ANS at room temperature for 24 hours. Determination of the free ANS in the equilibrium dialysis revealed the association of 2.6 moles of ANS/mole of lecithin; no change was observed after longer periods of dialysis. A similar value was obtained by centrifugal method which assumes additivity of refractive index (Olins and Warner, 1967). In this method, the lecithin–ANS complex obtained after the dialysis was diluted 1:1 with 0.079 M ANS (equilibrium concentration) and placed in a synthetic boundary cell. The boundary was formed by layering ANS solution of equilibrium concentration over the lecithin–ANS solution so that free ANS would not contribute to the refractive index gradient at the boundary. The rotor speed was kept at 4059 rpm, and the schlieren peak area which is proportional to refractive index contribution of the complex was measured. To determine the fraction of the area due to the lecithin component, synthetic boundary was also formed by layering buffer over the lecithin dispersion of the same concentration (6.4 mg/ml). The area produced by ANS solution of the concentration of 50 µmole/ml was obtained in the same manner by layering 0.05 M ANS over 0.1 M ANS. Since in all centrifugal runs the same bar angle (80°) was used and the boundaries formed at the same radial distance did not move significantly, the number of µmoles of ANS complexed with lecithin was obtained by dividing the difference between the areas of the lecithin–ANS complex and the lecithin sol alone by the area corresponding to 1 µmole of ANS. The molar ratio of ANS to lecithin in the complex was calculated to be 2.4.

The partial specific volume of the lecithin–ANS complex with a molar ratio of ANS to lecithin of 2.4 was approximated from the apparent specific volumes of ANS, 0.730, and of lecithin dispersions, 0.972, determined by pycnometry at 20°; a value of 0.853 was obtained. In this computation, additivity of molar volumes was assumed as is customary with protein–sodium dodecyl sulfate complex (Carusi and

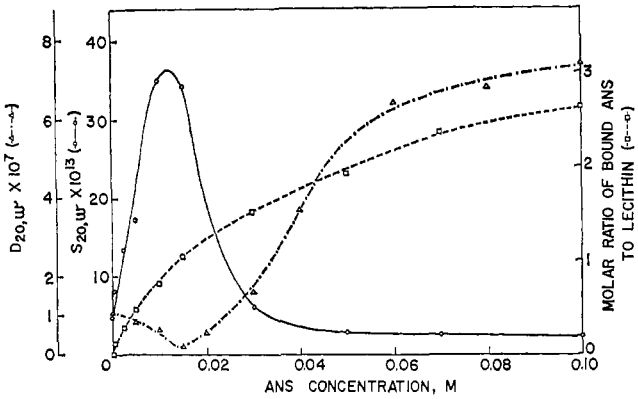

FIGURE 8: Effect of ANS concentration on the sedimentation coefficient and diffusion coefficient of lecithin–ANS complex and on the molar ratio of bound ANS to lecithin. Solutions containing egg lecithin dispersions (5 mg/ml) and ANS of various concentrations were dialyzed against ANS solutions of the same concentrations at room temperature for 20 hr. A blank was run for each concentration of ANS. The medium used for the equilibrium dialysis was 0.1 M sodium chloride–0.02 M phosphate buffer–0.05% EDTA (pH 7.0). After the dialysis the sedimentation coefficient and diffusion coefficient of lecithin–ANS complex were determined. The molar ratio of bound ANS to lecithin was determined as described in the section of Materials and Methods. All values of sedimentation coefficient and diffusion coefficient plotted are from single determinations. The values of the molar ratio are the means of three separate experiments. The standard deviation of the values from the separate experiments varied from 2 to 10%; the deviation was generally large in the experiments with high initial concentrations of ANS due to the difficulty encountered in accurate determination of small concentration differences.

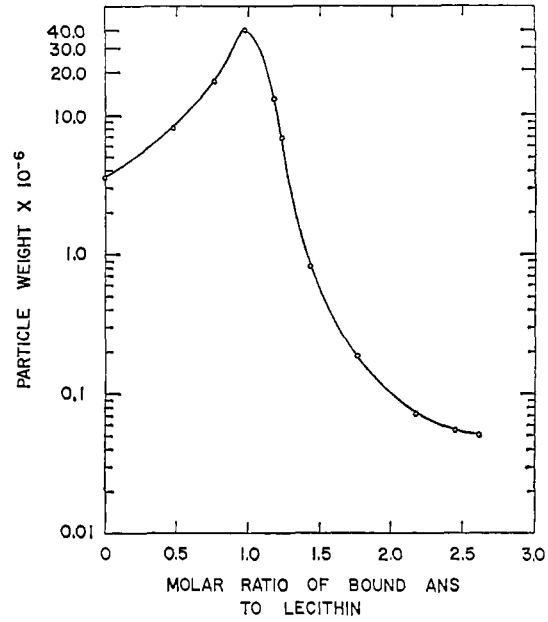

FIGURE 9: Relationship between the particle weight of lecithin–ANS complex and the molar ratio of bound ANS to lecithin. The particle weight of lecithin–ANS complex was computed from the sedimentation coefficient and diffusion coefficient given in Figure 8 and from the partial specific volume approximated in the same manner as described in the text and was plotted as a function of the molar ratio of bound ANS to lecithin.

Sinsheimer, 1963; Hersh and Schachman, 1958) and with lipopolysaccharide–sodium dodecyl sulfate complex (Olins and Warner, 1967). The diffusion coefficient ($D_{20,w}$ = 7.37 × 10^{-7}) was estimated from the area and peak height of boundaries (Lungren and Ward, 1951) produced in the synthetic boundary run described for the measurement of the extent of association of ANS with the lecithin. A horizontal line was obtained upon plotting diffusion coefficients against time. Computation of the particle weight of the lecithin–ANS complex from the partial specific volume, the diffusion coefficient, and the sedimentation coefficient/($s_{20,w}$ = 2.44 × 10^{-13} S) gave a value of 5.4 × 10^4. This particle weight was considered to be an approximate value because of a number of assumptions involved. The average particle weight of the original lecithin dispersions was computed to be approximately 3.5 × 10^6 from the apparent specific volume of the dispersions, 0.972, the diffusion coefficient ($D_{20,w}$ = 1.10 × 10^{-7}), and the sedimentation coefficient ($s_{20,w}$ = 4.71 × 10^{-13} S). Since the contribution of the lecithin component to the particle weight of the lecithin–ANS complex was approximately 2.6 × 10^4, it was apparent that 0.1 M ANS disrupted the original lecithin particles into small units in which the number of lecithin molecules present was less than 0.01 the number in the original particle.

To obtain some indication of the nature of the disruption of the lecithin particles by ANS, lecithin dispersions (5 mg/ml) and ANS of various concentrations were subjected to equilibrium dialysis for 20 hr at room temperature. The relationship of the sedimentation coefficient and diffusion coefficient of lecithin–ANS complex and the molar ratio of bound ANS to lecithin was determined (Figure 8). Upon increase in the concentration of ANS, the sedimentation coefficient of the complex progressively increased, while the diffusion coefficient decreased. At an ANS concentration of approximately 0.012 M, the maximum sedimentation coefficient and minimum diffusion coefficient were attained. At this concentration the molar ratio of bound ANS to lecithin was roughly one. Further increase in the ANS concentration decreased the sedimentation coefficient at first very rapidly, then gradually. The diffusion coefficient was increased progressively upon increase in the ANS concentration above 0.012 M. The relationship between the molar ratio of bound ANS to lecithin and the particle weight of lecithin–ANS complex computed from the sedimentation coefficient, the diffusion coefficient, and the apparent specific volume is also given (Figure 9). It was apparent that an initial increase in the bound ANS increased the particle weight of the complex leading to maximum aggregation upon equimolar association of ANS with lecithin. Further increase in the bound ANS disintegrated the aggregates into progressively smaller units. It was noted that the aggregation of the complex often produced a gel when lecithin dispersions with a sedimentation coefficient greater than 5.0 were used; gelation was observed only in the region of bound ANS to lecithin molar ratio of one. When ANS concentration was greater than 0.03 M, the sedimentation coefficient of lecithin–ANS complex was not influenced by the initial sedimentation coefficient of lecithin dispersions used.

It was suspected that ammonium ANS at high concentrations may not exist as a monomer (mol wt 315) but rather in polymeric forms and that the ability of bound ANS molecules to associate intermolecularly might have facilitated the disruption of lecithin dispersions at high ANS concentrations. Determination of the molecular weight indeed revealed that ANS in a 0.1 M solution was present as a tetramer. Using Archibald's method of approach to sedimentation equilibrium, an average molecular weight of 1270 was obtained at the meniscus of the cell and 1560 at the bottom

TABLE V: Molecular Weight of NH_4ANS at the Concentration of 0.1 M.

Method	Mol Wt[a]
Archibald's method of approach to sedimentation equilibrium	1270[b]
	1560[c]
Sedimentation equilibrium	1300[d]

[a] An apparent specific volume of 0.73 determined by pycnometry at 20° was used. The solvent was 0.1 M sodium chloride–0.02 M phosphate buffer–0.05% EDTA (pH 7.0). [b] The average of 1220 and 1320 obtained at the meniscus of the analytical centrifuge cell 16 and 32 min, respectively, after a maximum speed of 39,460 rpm was reached. Acceleration time was 4 min 10 sec. [c] The average of 1540 and 1590 obtained at the bottom of the cell after the same intervals given for the determination at the meniscus. [d] Sedimentation equilibrium run was made at 12,590 rpm. The molecular weight was determined as described by Richards *et al.* (1968). The slope of the linear portion of the log c *vs.* r^2 plot obtained after 58-hr centrifugation at 20° was 5.33×10^{-3}. A longer period of centrifugation, 68 hr, did not shift the plot.

(Table V). The higher value at the bottom probably reflected the presence of larger molecular aggregates. Determination of the ANS molecular weight was also performed by the sedimentation equilibrium method at a relatively low speed, 12,590 rpm, to avoid a steep concentration gradient of ANS in the solution column (0.28 cm in height) so that the deviation in the molecular weight in the column at equilibrium would be minimized. The plotting of log c *vs.* r^2 after 58 hr centrifugation gave a straight line except at the bottom of the cell. No change in the entire plot was observed after 68 hr centrifugation indicating that an equilibrium had been attained. A slight upward deviation of the plot at the bottom of the cell indicated some heterogeneity. The molecular weight calculated from the slope of the linear portion of the plot near the top of the cell was close to four times the monomer molecular weight of 315 (Table V). At equilibrium, the concentrations of ANS estimated from fringe numbers were 0.098 M at the meniscus and 0.102 M at the bottom.

Discussion

In the present study, the ultimate consequence of the interaction of NH_4ANS with LDL and HDL was the disruption of these lipoproteins into protein and lipid fractions. The lipid fraction was further disrupted into phospholipid-rich and neutral lipid-rich fractions. It was apparent that ANS at high concentrations disrupted both lipid protein and lipid–lipid bonds.

The displacement or release of virtually all the protein from LDL seemed to occur immediately upon addition of ANS to LDL to a concentration of 0.1 M. No appreciable increase in the size of the protein peak (s_{0bsd} = 14.5 S) was observed when the mixture was analyzed after a prolonged standing period. The LDL protein released required the presence of ANS to remain soluble. A series of dialysis experiments with 0.1% LDL protein against buffer containing varying concentrations of ANS at 4° indicated that the protein starts to precipitate when free ANS concentration is reduced to 15 mM and is completely precipitated at a concentration of 1–2 mM. Fifty per cent of the protein precipitated at a concentration of approximately 6 mM. It is likely that apolipoproteins in general may have nonpolar regions which associate with nonpolar lipid residues. The existence of such regions may be suggested by the persistence of apo-HDL to bind phospholipids under conditions which interfere with ionic association (Scanu, 1967) and from our preliminary results on the binding of more hydrophobic fluorescent dyes, such as perylene and methylanthracene to various apolipoproteins. It is possible that with the ANS ring system occupying hydrophobic sites and the sulfonate group directed into the aqueous environment, the effective hydrophobicity of the regions would be decreased, thus preventing extensive intermolecular association and precipitation of the proteins. This phenomenon may be analogous to detergent solubilization of lipid-free LDL protein obtained by solvent extraction of native or succinylated LDL (Gotto *et al.*, 1968a,b; Scanu *et al.*, 1968; Shore and Shore, 1967; Granda and Scanu, 1966). In contrast to LDL, only a very minor portion of HDL protein was precipitated when ANS was removed by dialysis against phosphate buffer of pH 7.0; the precipitate dissolved readily in 0.1 M ANS. It was previously reported that apo-HDL obtained by extraction with ethanol–diethyl ether (3:2, v/v) at −10° was only partially soluble at this pH (Scanu, 1966) while the fresh apo-HDL obtained by the extraction with ethanol–diethyl ether (1:3, v/v) at −20° gave completely clear solutions in neutral 0.9% saline solution (Sodhi and Gould, 1967).

Disruption of the lipid moieties of LDL and HDL into neutral lipid-rich and phospholipid-rich fractions suggests penetration of ANS between lipid–lipid interfaces with subsequent liberation of the phospholipid-rich surface layer from the total lipid moiety. However, as shown by the ultracentrifugal analysis of LDL in 0.1 M ANS, the cleavage of total lipid fraction was time dependent; the appearance of a floating peak which is likely to correspond to the neutral lipid-rich fraction required a considerable standing period. This was in contrast to the almost immediate cleavage of the lipoproteins into protein and total lipid fractions. In density gradient centrifugation, the separation of phospholipid-rich and neutral lipid-rich fractions seemed to be caused by gradual cleavage of total lipid fractions during density gradient centrifugation and subsequent flotation of neutral lipid-rich fraction to the top of the density gradients. Increase in the turbidity of neutral lipid-rich fractions after prolonged storage appeared to be caused by coalescence of the neutral lipids which became exposed upon loss of phospholipids. It is apparent that phospholipids are essential for the dispersion of neutral lipids in aqueous media and that the neutral lipids cannot be stabilized by ANS alone.

Some insight into the nature of lipid disruption by ANS was obtained by the study of the interaction of ANS with lecithin. Sonified lecithin dispersions are believed to exist in a lamellar-type structure (Chapman *et al.*, 1968) and have a minimum particle weight of approximately 3×10^6 (Scanu, 1967). In the presence of 0.1 M ANS, these large particles were disintegrated into ANS–lecithin units with a particle weight of approximately 5.5×10^4. Since the ANS:lecithin molar ratio of the disintegrated units was approximately 2.4, the disruption of the lamellar lecithin structure was apparently due to penetration by a large number of ANS molecules.

Although no definite information can be given at the present concerning the mechanism of ANS penetration and

subsequent disruption of lecithin particles, a sequence of events can be speculated from the study of the relationship between the particle weight of lecithin–ANS complex and the bound ANS:lecithin molar ratio (Figure 9). It has previously been shown that the binding of a variety of ionic fluorescent dyes to bovine serum albumin as well as to detergent molecules involves electrostatic interaction in addition to nonpolar interaction (Laurence, 1951; Flanagan and Ainsworth, 1968). Furthermore, strong enhancement of ANS fluorescence by oleylamine, cetyltrimethylammonium bromide, and lecithin dispersions suggested the possible binding of ANS to basic groups of the long-chain compounds (Hasselbach and Heimberg, 1970). Since in the present study the particle size of the lecithin–ANS complex reaches a maximum at the molar ratio of one, the association of ANS with lecithin is likely to involve an electrostatic interaction between the sulfonate group of ANS and the choline nitrogen of lecithin. Such an interaction at a molar ratio of one would minimize the surface polar quality of the complex, thus enhancing nonpolar interactions between complexes which eventually are converted to large aggregates. Although the electrostatic interaction between ANS sulfonate group and lecithin zwitterionic polar head leaves a net negative charge on the polar head, the maximum aggregation at the ratio of one suggests that the negative charge on the phosphate group cannot exert repulsive effects due possibly to the change in solvent environment and to steric factors. The electrostatic interaction between ANS and lecithin also limits the penetration of ANS molecules, which have relatively small dimension, into the hydrophobic interior of the lecithin particles. Furthermore, when the ANS:lecithin molar ratio of the complex is greater than one, the excess ANS may exist in association with the bound ANS in such a way that the sulfonate groups of the excess ANS are exposed into the media and have repulsive effects. This intermolecular association and the limited penetration of the ANS molecules may well explain the formation of lecithin–ANS units having a small particle weight or a greatly increased surface area to volume ratio at an ANS concentration of 0.1 M. Although the above speculation has to be substantiated by further study, the system described may be somewhat similar to the interaction of *N*-myristoylhistidine with cetyltrimethylammonium bromide (Gitler and Ochoa-Solano, 1968). At the molar ratio of 1, the mixture produced maximum turbidity due possibly to complete charge neutralization. When the molar ratio is deviated from 1, the appearance of net positive or negative charge reduced the turbidity. Although no systematic study has previously been made on the interaction of zwitterionic phospholipids with alkyl detergent, the study may be of great importance to provide information on the availability of phospholipid charged groups for electrostatic interaction.

The disruption of LDL and HDL may be initiated by the penetration of ANS into spaces between lipids with polar head groups and into lipid–protein interfaces. In the presence of a high concentration of ANS, binding of a large number of ANS molecules to the phospholipids may result in rearrangement of the phospholipid-rich surface layer, thus facilitating ANS penetration into the lipid–protein interface and eventual liberation of the protein. The lipid moiety could then rearrange further leading to gradual disruption into phospholipid-rich and neutral lipid-rich fractions. We have noted that the phospholipid particles are disrupted very rapidly in the presence of ANS at high concentrations. Ultracentrifugal analysis carried out immediately after mixing the phospholipid dispersions and ANS showed that the disruption was completed by the time the boundary was resolved. Thus, the time-dependent cleavage of the total lipid moiety of lipoproteins may reflect the inability of ANS to interact with neutral lipids or the inefficiency of ANS penetration into the neutral lipid–phospholipid interface.

While high concentrations of ammonium ANS promoted the disruption of the lipoproteins, lower ANS concentrations led to the formation of soluble LDL aggregates. Aggregation was not observed with HDL. It is possible that phospholipid polar groups are essential to maintain the solubility of LDL in aqueous solution. When the phospholipids are associated with ANS at an equimolar ratio, the contribution of the phospholipids to the solubility would be minimized. This would enhance intermolecular association of the LDL–ANS complex.

Ionic and nonionic detergents have long been known to disrupt lipid–lipid and lipid–protein bonds of plasma lipoproteins (Gurd, 1960). High concentrations of fatty acids, saponins, cholate, etc., displaced a portion of the lipids from the lipoproteins. Recently Gotto *et al.* (1969) showed that sodium decyl sulfate disrupts β-lipoprotein extensively. The protein fraction obtained by the disruption and by subsequent centrifugation was in association with approximately 50% each of the phospholipids and free cholesterol of the original lipoproteins. However, more recently Simons and Helenius (1970) reported in their communication that LDL protein can be obtained free from lipids when LDL is treated with 0.2 M sodium dodecyl sulfate and subjected to gel filtration on Sepharose 4B using a buffer containing 0.1 M sodium dodecyl sulfate. Apparently sodium dodecyl sulfate and ANS disrupt lipoproteins in an analogous manner and allow the separation of apolipoproteins without exposure to organic solvents. It is possible that LDL disrupted by sodium dodecyl sulfate can only be fractionated successfully by gel filtration in the presence of the detergent and not by centrifugation or density gradient centrifugation. We have noted that unlike NH_4ANS, sodium dodecyl sulfate, which produces large micelles, concentrates as a band in density gradient centrifugation. This phenomenon would change the state of complex equilibrium between bound and unbound detergent and hence the physical state of the disrupted LDL components.

Although ANS of high concentrations (10^{-1}–10^{-3} M) was used in the present study to effect the disruption of plasma lipoproteins, the disruptive effect of ANS does not preclude its use as a fluorescent probe for the study of plasma lipoproteins at the concentrations (10^{-4}–10^{-6} M) customarily used in fluorescent studies (Stryer, 1965). Indeed a recent fluorescent study with ANS (Hart *et al.*, 1970) revealed that high-density lipoprotein (HDL_2) binds approximately 300 molecules of ANS while the apolipoprotein has three binding sites, thus indicating the major contribution of lipids for the binding of ANS to the lipoprotein. A study is currently in progress in our laboratory to provide further information on the nature of the interaction of ANS and other hydrophobic probes with lipoproteins and various lipid dispersions.

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References

Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. (1952), *J. Biol. Chem. 195*, 357.

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

Bragdon, J. H., Havel, R. J., and Boyle, E. (1956), *J. Lab. Clin. Med. 48*, 36.

Brand, L., Gohlke, J. R., and Rao, D. S. (1967), *Biochemistry 6*, 3510.

Carusi, E. A., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 7*, 388.

Chapman, D., Fluck, D. J., Penkett, S. A., and Shipley, G. G. (1968), *Biochim. Biophys. Acta 163*, 255.

Chung, J., and Nishida, T. (1967), *J. Lipid Res. 8*, 631.

Edelman, G. M., and McClure, W. O. (1968), *Accounts Chem. Res. 1*, 65.

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Flanagan, M. T., and Ainsworth, S. (1968), *Biochim. Biophys. Acta 168*, 16.

Gitler, C., and Ochoa-Solano, A. (1968), *J. Amer. Chem. Soc. 90*, 5004.

Gotto, A. M., Levy, R. I., and Fredrickson, D. S. (1968a), *Biochem. Biophys. Res. Commun. 31*, 151.

Gotto, A. M., Levy, R. I., and Fredrickson, D. S. (1968b), *Lipids 3*, 463.

Gotto, A. M., Levy, R. I., Lindgren, F. T., and Fredrickson, D. S. (1969), *Biochim. Biophys. Acta 176*, 667.

Granda, J. L., and Scanu, A. (1966), *Biochemistry 5*, 3301.

Gurd, F. R. N. (1960), *in* Lipid Chemistry, Hanahan, D. J., Ed., New York, N. Y., Wiley, p 260.

Hanahan, D. J., Dittmer, J. C., and Warashina, E. (1957), *J. Biol. Chem. 228*, 685.

Hart, C. J., Leslie, R. B., and Scanu, A. M. (1970), *Chem. Phys. Lipids 4*, 367.

Hasselbach, W., and Heimberg, K. W. (1970), *J. Membrane Biol. 2*, 341.

Hersh, R. T., and Schachman, H. K. (1958), *Virology 6*, 234.

Janado, M., and Nishida, T. (1965), *J. Lipid Res. 6*, 331.

Janado, M., and Nishida, T. (1967), *Agr. Biol. Chem. 31*, 802.

Laurence, D. J. R. (1952), *Biochem. J. 51*, 168.

Levy, R. S., Lynch, A. C., McGee, E. D., and Mehl, J. W. (1967), *J. Lipid Res. 8*, 463.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lungren, H. P., and Ward, W. H. (1951), *in* Amino Acids and Proteins, Greenberg, D. M., Ed., Springfield, Ill., C. C Thomas, p 353.

Margolis, S., and Langdon, R. G. (1965), *J. Biol. Chem. 241*, 469.

Mauldin, J., and Fisher, W. R. (1970), *Biochemistry 9*, 2015.

McClure, W. O., and Edelman, G. M. (1966), *Biochemistry 5*, 1908.

Minari, O., and Zilversmit, D. B. (1963), *Anal. Biochem. 6*, 320.

Olins, A. L., and Warner, R. C. (1967), *J. Biol. Chem. 242*, 4994.

Ouchterlony, O. (1953), *Acta Pathol. 32*, 231.

Parker, F., and Peterson, N. F. (1965), *J. Lipid Res. 6*, 455.

Rhodes, D. N., and Lea, C. H. (1957), *Biochemistry 65*, 527.

Richards, E. G., Teller, D. C., and Schachman, H. K. (1968), *Biochemistry 7*, 1054.

Rosenberg, R. M., and Klotz, I. M. (1960), *in* Analytical Methods of Protein Chemistry, Vol. 2, Alexander, P., and Block, R. J., Ed., New York, N.Y., Pergamon Press, p 133.

Scanu, A. (1966), *J. Lipid Res. 7*, 295.

Scanu, A. (1967), *J. Biol. Chem. 242*, 711.

Scanu, A., Pollard, H., and Reader, W. (1968), *J. Lipid Res. 9*, 342.

Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.

Schwenk, E., and Werthessen, N. T. (1952), *Arch. Biochem. Biophys. 40*, 344.

Shore, B., and Shore, V. (1967), *Biochem. Biophys. Res. Commun. 28*, 1003.

Simons, K., and Helenius, A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 59.

Sodhi, H. S., and Gould, R. G. (1967), *J. Biol. Chem. 242*, 1205.

Stryer, L. (1965), *J. Mol. Biol. 13*, 482.

Turner, D. C., and Brand, L. (1968), *Biochemistry 7*, 3381.

Van Handel, E., and Zilversmit, D. B. (1957), *J. Lab. Clin. Med. 50*, 152.

Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, 31.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239*, 1415.

Zak, B., Luz, D. A., and Fisher, M. (1957), *Amer. J. Med. Technol. 23*, 283.

Zilversmit, D. B. (1965), *J. Clin. Invest. 44*, 1610.