Location of Aromatic Amino Acid Residues in Bovine Serum High-Density Lipoprotein†

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ABSTRACT: The location of tyrosine and tryptophan residues in intact bovine serum high-density lipoprotein (BHDL) and in the delipidated protein component (apo-BHDL) was determined by solvent perturbation of the chromophores, using difference absorption spectroscopy. The changes in the chromophore environment upon delipidation of the lipoprotein were confirmed by spectrophotometric titration of the tyrosine residues and by changes in the intrinsic fluorescence spectra. It was found that in BHDL, 75% of the tyrosine residues and 40% of the tryptophan residues are exposed to solvent, whereas in apo-BHDL, 100% of the tyrosine and nearly 70% of the tryptophan residues are exposed. Comparison of these results with data of other investigators indicates that BHDL has, on the average, a larger proportion of its aromatic residues accessible to solvent than soluble proteins of comparable size, and that apo-BHDL must have a very loose or extended configuration in solution, markedly different from globular proteins. The present results suggest a model of BHDL where most of the protein is located on the surface of the lipoprotein in an extended form, and imply the existence of specific protein–lipid interactions rather than bulk hydrophobic interactions.

Protein configuration and the precise nature of protein–lipid interactions in high-density serum lipoproteins (HDL)[1] are very important problems which relate to the questions of structure and interactions in other, more complex lipoprotein systems such as biological membranes.

Although several facts have been established about the structure of human HDL, for example, predominantly nonpolar protein–lipid interactions, a high average α-helical structure of the protein components (60–70% α helix, Scanu and Hirz, 1968), and surface location of most polar amino acid residues (Scanu *et al.*, 1968), there is no general agreement on a model for the protein conformation in the intact lipoprotein. Electron microscopy suggests spherical shapes with subunit structure for human and other mammalian HDL's (Forte and Nichols, 1972); the nature of the subunits is not clear, however; they could represent lipid, protein, or lipoprotein entities (Scanu and Wisdom, 1972). Some authors have proposed a model for HDL where globular protein subunits are partially submerged in lipid (Scanu, 1967); others favor the view that HDL consists of several complete lipoprotein globules, each having a lipid core and a protein envelope (Nichols, 1969; Gotto, 1969). In addition to these two models, small-angle X-ray scattering results (Scanu and Wisdom, 1972) and trypsin and phospholipase digestion experiments (Camejo, 1969), suggest a structure for HDL where most of the protein and polar lipid groups are localized on the surface of the particle, which has a radially uniform, electron-deficient interior.

The objective of this work was to obtain some experimental evidence that would help elucidate the structure of the protein component of HDL, and to gain some new information on the structure of the apolipoprotein in solution. The main approach consisted of locating the aromatic amino acid residues in BHDL and in apo-BHDL by the solvent perturbation technique of Herskovits and Laskowski (1962) and Herskovits and Sorensen (1968a,b). Because the aromatic amino acid residues are among the most hydrophobic groups in proteins (Goldsack, 1970; Nozaki and Tanford, 1971) their location in a lipoprotein may give an indication of the distribution of other nonpolar groups, as well.

The solvent perturbation technique, used in this work, is based on the fact that changes in the composition of solutions cause small shifts in the absorption spectra of chromophoric solutes. These spectral shifts have been attributed to changes in solvation energies of the ground and excited states of the chromophore. In other words, the shifts result from complex but short-range interactions of chromophore and solvent. The interaction distances are of the order of a few ångstroms, small, compared to the dimensions of most proteins. On this basis, Herskovits and Laskowski (1961, 1962) developed the solvent perturbation method to distinguish between "buried" and "exposed" tyrosine and tryptophan residues in proteins.

Materials and Methods

BHDL and apo-BHDL were prepared, and protein was determined, as described in a previous paper (Jonas, 1972a). *N*-Acetyl ethyl esters of tyrosine and tryptophan, used in evaluating the performance of the Cary 15 recording spectrophotometer in the 0–0.1 absorbance scale, were purchased from Mann Research Laboratories. Methanol, ethylene glycol, glycerol, and all other solvents and reagents were of the highest purity available commercially. All solutions were filtered through Millipore filters before use.

Difference absorption spectra in the solvent perturbation experiments were recorded with a Cary 15 spectrophotometer following essentially the procedure of Herskovits and Laskowski (1962), and Herskovits (1967). Matched cylindrical tandem double cells of 1-cm path length in each of the two

† From the Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received June 15, 1973.* This work was initiated in Professor G. Weber's laboratory with the support of U. S. Public Health Service Grant GM-11223 from the National Institutes of Health, and was completed in the Max Planck Institut für Medizinische Forschung, Abteilung für Chemie, Heidelberg, Germany, under a North Atlantic Treaty Organization Postdoctoral Science Fellowship, 1973.

[1] Abbreviations used are: HDL, high-density serum lipoproteins; BHDL, bovine high-density lipoprotein; apo-HDL, delipidated protein components of HDL; apo-BHDL, delipidated BHDL.

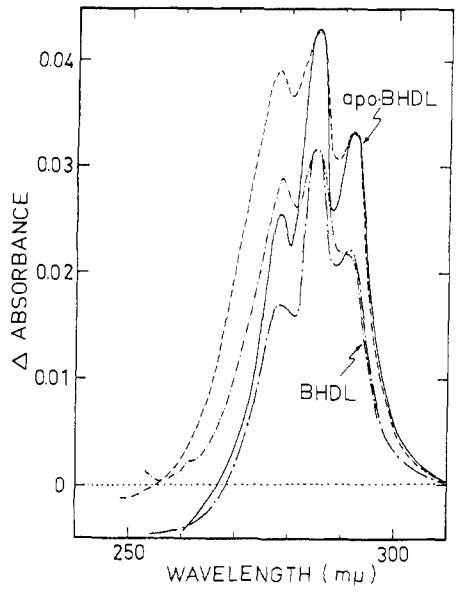

FIGURE 1: Difference absorption spectra of BHDL and apo-BHDL exposed to 20% ethylene glycol. Experimental spectra for BHDL (-·-·-·-·-) and apo-BHDL (- - - -), obtained in 0.01 M potassium phosphate buffer, 0.1 M KCl, and 0.025% EDTA (pH 6.8). The experimental spectra were normalized for the same protein concentration of 5.0×10^{-5} M using 28,000 as the protein molecular weight. Calculated spectra for BHDL (—·—) and for apo-BHDL (——), for 5.0 exposed tyrosine and 1.2 exposed tryptophan residues, and for 6.3 exposed tyrosine and 2.0 exposed tryptophan residues, respectively.

compartments were manufactured by Pyrocell, Inc., New York, N. Y. Solutions of BHDL and apo-BHDL in 0.01 M potassium phosphate buffer, 0.1 M in KCl, and 0.025% EDTA (pH 6.8), had final absorbance values between 1.5 and 2 ODU at 280 mμ. The perturbants were 20% by volume in the final solutions. Every two or three measurements the base line was checked and readjusted, if necessary, to "zero," with the set of multipodes in the spectral region from 350 to 240 mμ. The best spectral resolution together with the lowest noise level was attained, for our instrument, at a dinode setting of 3 and a sensitivity of 3 to 4. During the measurements maximum slit widths did not exceed 1 mm, and Beer's law was obeyed at all wavelengths of interest. The experimental errors of this technique were estimated by measuring difference spectra between identical bovine serum albumin solutions (crystalline, Armour Pharmaceutical Co. product, 2 ODU at 280 mμ) in the sample and reference cells. In individual measurements, the maximum errors were 20–30%; however, the final results reported in this work are the average of at least ten measurements, with a total error not higher than 5%. Results were calculated using difference absorbance values (ΔA) at 291–293 and 286–288 mμ and the difference molar extinction coefficients for model compounds, in the presence of solvent perturbants, given by Herskovits and Sorensen (1968a).

Spectrophotometric titrations of tyrosine residues (Tanford, 1961) in BHDL and apo-BHDL in 0.1 M NaCl at concentrations around 6×10^{-6} and 2.6×10^{-5} M, respectively, were carried out by manual additions of 0.10 and 1.0 N NaOH to 2.0 ml of sample solutions in 1-cm light-path absorption cells. The pH values were measured with a Corning pH-meter equipped with a glass electrode; absorbance increases were read at 295 mμ with a Zeiss PMQ II spectrophotometer. The number of phenol groups titrated was calculated from molar extinction coefficient data of Beaven and Holiday (1952):

TABLE 1: Molar Absorptivity Difference Data for BHDL and apo-BHDL in Three Solvents. Exposed Tyrosine and Tryptophan Residues.[a]

Solutions	$\Delta\epsilon_{286}$	$\Delta\epsilon_{292}$	Exposed Tyr	Exposed Trp
BHDL in 20% (v/v)				
Methanol	518	346	5.1	1.3
Ethylene glycol	627	449	5.0	1.2
Glycerol	587	420	4.8	1.2
Apo-BHDL in 20% (v/v)				
Methanol	684	501	6.3	1.9
Ethylene glycol	868	742	6.3	2.1
Glycerol	811	653	6.2	1.9

[a] Molar absorptivity values were calculated from difference absorbance data obtained for protein concentrations between 3.0×10^{-5} and 1.2×10^{-4} M, in 0.01 M potassium phosphate buffer, 0.1 M KCl, and 0.025% EDTA (pH 6.8). The molecular weight of the protein component was 28,000. Values given in the table are the average of 5–10 experimental observations. Exposed tyrosine and tryptophan residues were calculated from the molar absorptivity data and values for model compounds given by Herskovits and Sorensen (1968a).

$\epsilon = 2.33 \times 10^3$ M^{-1} cm^{-1} for tyrosine at 293.5 mμ in 0.1 N NaOH solution.

Intrinsic fluorescence spectra were obtained with the spectrofluorometer described by Weber and Young (1964), and were corrected for the spectral efficiency of the emission monochromator and photomultiplier response. Solutions of BHDL and apo-BHDL in 0.01 M potassium phosphate buffer (pH 6.8), 0.1 M KCl, and 0.025% EDTA had the same absorbance value at the excitation wavelength of 275 mμ.

The structural integrity of BHDL in 20% ethylene glycol, 20% glycerol, and alkaline solutions of pH 11.5–12.0 was determined by measuring sedimentation velocity coefficients of BHDL under those conditions, using a Beckman analytical ultracentrifuge, Model E-359. Sedimentation velocity coefficients were reduced to standard conditions ($s_{20,w}$, water solution at 20°) by employing density and viscosity values determined for the solvents. Densities were obtained by picnometry and viscosities were measured in a thermostated water bath using Cannon viscometers.

Results

Solvent Perturbation. Figure 1 shows a typical difference absorption spectrum of BHDL in the presence of 20% ethylene glycol measured against the lipoprotein solution without perturbant. The theoretical spectrum, calculated for 5.0 exposed tyrosine and 1.2 exposed tryptophan residues per protein subunit of 28,000 molecular weight, using data for model compounds given by Herskovits and Sorensen (1968a), agrees fairly well with the experimental spectrum in the wavelength region from 310 to 284 mμ, which includes the wavelengths of interest in the calculations. At shorter wavelengths, the theoretical spectrum deviates considerably from the experimental spectrum, a fact also observed by Herskovits and Sorensen (1968b) for various perturbants and proteins. The discrepancy is probably due to contributions to the protein difference spectra by groups other than tyrosine and tryptophan, which absorb at shorter wavelengths (*e.g.*, phenyl-

TABLE II: Per Cent Exposure of Tyrosine and Tryptophan Residues to Solvent in BHDL, Apo-BHDL, and in Various Proteins.[a]

Protein	Tyr Exposure (%)	Trp Exposure (%)
BHDL	75	40
Apo-BHDL	100	70
Aldolase[b]	40	20
L-Glutamate dehydrogenase[c]	31	40
Bovine fibrinogen[d]	33	33
Bovine serum albumin[b]	30	50
Pepsin[b]	60	50
Paramyosin[e]	55	
β-Lactoglobulin[f]		35
Carbonic anhydrases[g]		35
Lysozyme[h]		74

[a] Average values determined by solvent perturbation with perturbants of a mean diameter up to 6 Å. [b] Herskovits and Sorensen (1968b). [c] Cross and Fisher (1966). [d] Mihalyi (1968). [e] Riddiford (1966). [f] Herskovits (1965). [g] Lindskog and Nilsson (1973). [h] Williams *et al.* (1965).

alanine residues and amide bonds), and to differences in light scattering.

Results obtained using methanol, ethylene glycol, and glycerol as perturbants of BHDL absorption were identical within experimental error (5.0 ± 0.3 exposed tyrosine residues and 1.2 ± 0.1 exposed tryptophan residues). The values for all three solvents given in Table I indicate that the accessibility of the chromophores to solvents of mean diameters from 2.8 to 5.2 Å is very similar. Each protein subunit of BHDL (28,000 molecular weight) contains 6.4 tyrosine and 3.1 tryptophan residues (Jonas, 1972a). The average per cent exposure of these aromatic residues in intact BHDL is, therefore, 75 and 40%, respectively. These values are shown in Table II together with the data for apo-BHDL and with literature data for various proteins. BHDL has a diameter of about 100 Å, and has an overall rigidity similar to large globular proteins (Jonas, 1972b); however, BHDL has obviously a larger proportion of its aromatic residues exposed to solvent perturbation than most proteins studied by this method.

For the correct interpretation of solvent perturbation data, it is imperative that the total structure of the protein or lipoprotein under investigation remain unchanged in the presence of perturbants. From previous solvent perturbation experiments with proteins (see references in Table II), as well as from sucrose density gradient experiments, it is known that polyhydroxy compounds at concentrations around 20% do not affect significantly the structure of proteins. To prove that this is also the case with BHDL, I determined $s_{20,w}$ values for the lipoprotein in 20% ethylene glycol and 20% glycerol and found the same value of 5.0 ± 0.3 S as for BHDL in buffer alone. The conclusion is that the structure of BHDL is not affected significantly by either of these perturbants.

The same solvent perturbation experiments were performed on apo-BHDL as on intact BHDL. Figure 1 shows the experimental spectrum and the theoretical spectrum calculated for 6.3 ± 0.3 exposed tyrosine residues and 2.0 ± 0.2 exposed tryptophan residues per 28,000 molecular weight. Qualitatively, the agreement of experimental and calculated spectra is similar as in the case of BHDL, and the same comments

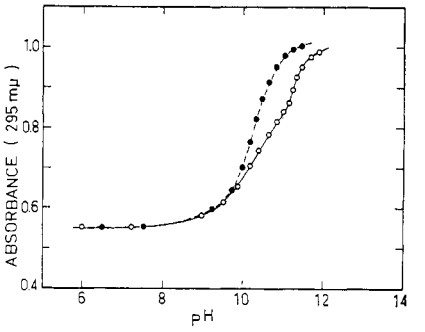

FIGURE 2: Spectrophotometric titration of tyrosine residues in BHDL (—○—○—) and apo-BHDL (- -●- -●-). Base titration of 6×10^{-6} M BHDL and 2.6×10^{-5} M apo-BHDL in 0.1 M NaCl.

hold. Table II gives the results as average per cent exposure of tyrosine and tryptophan residues. The results obtained for apo-BHDL with all three perturbants were the same and indicate almost total exposure of the aromatic residues to solvents of mean diameters up to 5.2 Å (Table I). Although in sodium dodecyl sulfate solutions apo-BHDL appears as a single main species of 28,000 molecular weight, in aqueous nondisaggregating solvents it exists in higher molecular weight forms (Jonas, 1972a). Under the conditions of the solvent perturbation experiments, apo-BHDL probably exists in aggregated forms, which, nevertheless, have most of the aromatic residues exposed to solvent.

Spectrophotometric Titration. Base titration of tyrosine residues in BHDL (Figure 2) reveals the presence of two types of residues. Four to five tyrosine residues titrate with a normal pK_a of 10.2, whereas the remaining two groups require somewhat higher pH values for ionization. Sedimentation velocity results show no major structural changes in BHDL at pH values of up to 11.5 or 12; therefore, it seems that the ionization of the two "buried" tyrosine residues involves some local structural rearrangements which do not affect significantly the overall structure of the lipoprotein. These results agree with the solvent perturbation data of 75% "exposed" tyrosine residues. A parallel experiment on apo-BHDL shows that all the tyrosine residues titrate with the same pK_a of 10.2, indicating an increased exposure of tyrosine residues to solvent upon delipidation of BHDL. The change corresponds to the 75–100% exposure change observed by solvent perturbation.

Fluorescence Spectra. The wavelength of maximum fluorescence for BHDL is 328 mμ and for apo-BHDL 338 mμ, in 0.01 M potassium phosphate buffer (pH 6.8) (Figure 3). The red shift in the fluorescence of the apolipoprotein and the 15% decrease in quantum yield with respect to intact BHDL are indications that upon delipidation tryptotophan residues become more exposed to aqueous solvent. It is well documented that proteins containing both tyrosine and tryptophan groups exhibit predominantly tryptophan fluorescence because of the much higher quantum yield of this chromophore (Teale and Weber, 1959); moreover, tryptophan fluorescence spectra undergo shifts of as much as 20–30 Å when thechromophore environment is changed from nonpolar to polar (Teale, 1960). This effect is essentially similar to the solvent perturbation effect on absorption spectra, above discussed.

The changes in fluorescence spectra from BHDL to apo-BHDL agree qualitatively with the solvent perturbation data that indicate a change from 40 to 70% exposure of tryptophan residues to solvent, upon delipidation.

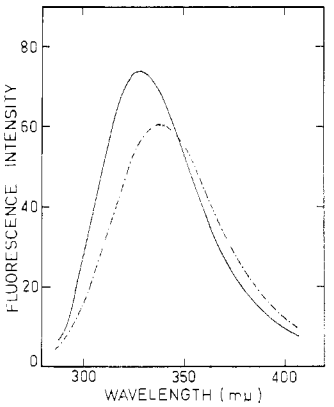

FIGURE 3: Corrected intrinsic fluorescence spectra of BHDL (——) and apo-BHDL (-·-·-·-). Spectra are in arbitrary fluorescence units. BHDL and apo-BHDL solutions in 0.01 M potassium phosphate buffer, 0.1 M KCl, and 0.025% EDTA (pH 6.8) had identical absorbance values at the exciting wavelength of 275 mμ.

Discussion

The current models of HDL structure impose some restrictions on the location of the hydrophobic amino acid residues in the lipoprotein. The model which proposes hydrophobic interactions of lipid with globular protein subunits (Scanu, 1967) would require most of the hydrophobic amino acid residues, including the aromatic ones, to be buried in the interior of the lipoprotein in contact with other nonpolar amino acid residues or with hydrocarbon portions of the lipids. In such a lipoprotein model the nonpolar amino acid residues would be expected to be better protected from solvent than in proteins of similar size. The quantitative solvent perturbation results presented in Table II show clearly that in BHDL the aromatic amino acid residues are considerably more exposed to solvent (75% Tyr and 40% Trp) than in aldolase (40% Tyr and 20% Trp) or in L-glutamate dehydrogenase (31% Tyr and 40% Trp). Even smaller and nonglobular proteins have, in general, less accessible aromatic residues than BHDL. Therefore, the present data do not support Scanu's (1967) model of HDL. It should be pointed out here that BHDL resembles more human HDL_2 than HDL_3 (Jonas, 1972a), but that in studies on the protein component of human HDL the two subclasses are used either in the unfractionated form or interchangeably because of the identity of their protein constituents.

In the discrete lipoprotein subunit model (Nichols, 1969; Gotto, 1969) the location of hydrophobic amino acid residues cannot be predicted readily; however, if the protein–protein interactions between subunits are assumed to be similar to interactions in subunit proteins, similar or slightly higher exposure of nonpolar amino acid residues would be expected for the lipoprotein as for comparable proteins, *e.g.*, aldolase and L-glutamate dehydrogenase. That this is not the case is indicated in Table II.

Although Scanu still seems to maintain his earlier model of HDL structure (Scanu, 1967), in a recent review (Scanu and Wisdom, 1972) this author mentions the results of small-angle X-ray scattering experiments on human HDL which indicate two well-defined secondary scattering maxima and one less well defined, which suggest a highly symmetrical particle. These results are interpreted in terms of a particle with an electron-deficient central core surrounded by an electron rich outer shell containing protein and polar lipid head groups. A similar interpretation about the structure of HDL on the basis of trypsin and phospholipase digestion experiments (Camejo, 1969) is criticized by Scanu and Wisdom (1972) because the structural integrity of the lipoprotein was not monitored and possibly not maintained in Camejo's experiments.

In this work, solvent perturbation data clearly indicate that, on the average, aromatic amino acid residues, classified as the first (Trp) and third or fourth (Tyr) most hydrophobic groups in proteins (Goldsack, 1970; Nozaki and Tanford, 1971), are considerably more exposed to solvent perturbation in BHDL than in most proteins so far investigated by this technique. The difference is especially marked between BHDL and aldolase or L-glutamate dehydrogenase (Table II), both large globular proteins that approach BHDL in size and shape. Smaller globular proteins, such as pepsin and serum albumin, have a higher degree of aromatic residue exposure to solvent than the larger proteins, probably as a consequence of a higher surface to volume ratio. Interestingly, bovine fibrinogen, which is a soluble but rather open protein, has the aromatic residues relatively well protected from solvent (33% Tyr and 33% exposed Trp). The available data are not sufficient to arrive at generalizations about the degree of aromatic residue exposure in relation to protein configuration, but it seems to depend on the size, and to a lesser extent on the shape and function of the protein. At any rate, the results obtained for BHDL and apo-BHDL indicate conclusively that in the lipoprotein and apolipoprotein, the protein configuration is quite different from that of most soluble proteins.

Unpublished results (Jonas, 1972) suggest that the protein structure of BHDL is in many ways very similar to human HDL. Polar amino acid residues are located on the surface of the particle: over 90% of the lysine residues is succinylated without any alterations in BHDL structure and essentially all ionizable groups in BHDL can be titrated completely and reversibly, in the pH range from 1 to 12, without loss of structural integrity of BHDL. BHDL, like human HDL, has from 60 to 70% α-helical structure in its protein component.

In view of the extensive surface exposure of polar amino acid residues and the high exposure of aromatic groups in BHDL and possibly in human HDL, the conclusion is that most of the protein component is located on the surface of the high-density lipoprotein. Therefore, the present results suggest strongly a model of HDL where the protein and the polar groups of lipids constitute the external layer of a particle with a nonpolar lipid core rather than models where considerable portions of the protein component are protected from the solvent, such as the globular protein subunit model of Scanu (1967) or the lipoprotein subunit model of Nichols (1969) and Gotto (1969). The subunit morphology of negatively stained HDL observed by electron microscope (Forte and Nichols, 1972) could be explained perhaps as the result of an uneven protein distribution on the surface of the lipoprotein or simply as an artifact of the staining and drying procedure.

Solvent perturbation data on apo-BHDL (100% exposed tyrosine and 70% exposed tryptophan residues, Table II) suggest a very open protein configuration of delipidated BHDL. It would seem that such results support the conclusion arrived at about the protein structure of HDL; however, one must keep in mind that the structure of the protein may change drastically once the lipids are removed. Nevertheless, the results on apo-BHDL are very interesting in that they point to a basic difference in the nature of soluble apolipoproteins and globular proteins. Globular proteins exist in compact configurations in solution, and when unfolded through some denaturation process which does not prevent intermolecular

interactions, tend to interact with each other and precipitate. This is apparently not the case with apo-BHDL which exists in solution in an extended configuration and forms aggregates of limited size. More detailed studies of the structure and properties of apo-BHDL or other apo-HDL's in solution may provide valuable information on the factors which determine their capacity to bind lipids.

Finally, the present work, with the conclusion that most of the protein is located on the surface of BHDL, suggests that protein–lipid interactions are not simply hydrophobic interactions of massive, nonpolar regions of the protein with nonpolar lipid domains, rather, it implies the existence of specific forces between lipids and probably a limited number of amino acid residues.

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Mechanism of Ion Escape from Phosphatidylcholine and Phosphatidylserine Single Bilayer Vesicles†

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ABSTRACT: The escape of $^{22}Na^+$ and $^{36}Cl^-$ from egg lecithin and ox-brain phosphatidylserine single-shelled vesicles has been measured at 4°. The mechanism of Cl^- escape involves diffusion across the phospholipid bilayer; this follows first-order kinetics. At pH 5.5 the first-order rate constant (k_1) is about three orders of magnitude higher than that for Na^+ diffusion and the enhanced Cl^- diffusion probably involves the covalent association of Cl^- ions and protons at the lipid–water interface. This is consistent with the finding that log k_1 is inversely proportional to pH. From a kinetic analysis of the Na^+ escape and from energetic considerations of the transport of ions across bilayers it is concluded that there are two contributions to the measured cation flux. These are diffusion across the bilayer and collision-induced rupture of the vesicles with concomitant release of the ions encapsulated in the internal cavities of the vesicles. The latter mechanism probably also involves aggregation and/or coalescence of the single-shelled vesicles to larger phospholipid structures; these effects occur more readily at higher temperatures. Since the relative contributions of the two mechanisms are unknown, the experimental data for Na^+ cannot be expressed as intrinsic bilayer permeabilities. In contrast, the Cl^- permeabilities seem to represent intrinsic values since at acid pH the diffusion is so fast that the contribution of the collision mechanism is negligible.

Phospholipid bilayers are now considered to play an important part in the structure and function of biological membranes (Stoeckenius and Engelman, 1969; Hendler, 1971; Phillips, 1972) and hence the properties of these bilayers in various model systems have been studied intensively during the past decade. In particular, aqueous dispersions of egg-yolk lecithin, both sonicated and unsonicated, have found wide application as a lipid bilayer model in biochemical and biophysical studies. Bangham *et al.* (1965) and Papahadjopoulous and Watkins (1967) used aqueous dispersions of egg lecithin to study ion flux rates across lipid bilayers. Radioactive ions were incorporated in egg lecithin particles which

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