Biochimica et Biophysica Acta, 552 (1979) 89—102

BBA 78317

SMALL ANGLE X-RAY SCATTERING FROM THE INNER AND OUTER MEMBRANES FROM *ESCHERICHIA COLI*

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(Received August 2nd, 1978)

Key words: Small angle X-ray scattering; Membrane fraction; (Escherichia coli)

Summary

Small angle X-ray data from purified forms of inner or cytoplasmic and outer membranes from *Escherichia coli* have been obtained and appear to be qualitatively similar. Transitory changes are apparent in the circularly averaged X-ray profiles from inner membrane. Such results could be due to the loss or denaturation of peripheral membrane proteins. Some partially dried forms of outer membrane are partly ordered and produce diffraction patterns which support an underlying bilayer structure. An extra light membrane fraction which results from membrane preparations utilizing a French pressure cell for spheroplast disruption has been characterized and shown to be similar to inner membrane. The purified membranes produce small angle X-ray diffraction patterns which are much different from those of lipid dispersions and the differences are attributable to the high protein content of the intact membranes. While the small angle X-ray region may be useful for characterizing the membrane preparations, the paucity of detail in the diffraction pattern suggest that it will be of little value in describing the complex underlying membrane structure.

Introduction

Many of the current ideas on membrane structure are based on diffraction data obtained from model or reconstituted lipid systems. However in several instances X-ray diffraction studies have been carried out directly on the cellular membrane system. This X-ray scattering can be roughly divided into two regions. The high angle region corresponding to real spacings ranging from 3 to 6 Å contains information from the packing of hydrocarbon chains in lamellar arrangements. In most cases, these are the only ordered components in a mem-

brane system. The small angle region of the X-ray scattering, corresponding to spacings greater than 10 Å, is a resultant of all the material present in the membrane. In systems containing mainly lamellar lipid, the small angle X-ray region is dominated by diffraction from the juxtaposed electron dense head group region of phospholipids in a bilayer arrangement.

The cytoplasmic and outer membrane systems from *E. coli* represents a particularly attractive source of material for X-ray diffraction studies. A number of mutants with altered lipid metabolism have been isolated and characterized (for a review, see ref. 1). Such mutants permit extensive controlled changes in the lipid composition of membrane specimens. In addition, temperature-dependent phase transitions for *Escherichia coli* membranes have been characterized by monitoring the high angle X-ray diffraction peak at 4.15 Å, the so-called hexagonal close packed reflection from the fatty acyl chains [2—5]. However, relatively little attention has been paid to the small angle X-ray scattering from *E. coli* membranes and in this report we describe such data from several preparations of vesicles of purified inner and outer membranes from *E. coli*. Similar X-ray diffraction studies have been done on membranes from *Acholeplasma laidlawii* [6]. These results showed that purified microbial membranes do indeed produce a sharp 4.15 Å reflection associated with close hexagonal fatty acid chain packing [6]. But of equal importance was the fact that the diffuse small angle maxima which were observed could be altered in a predictable fashion by supplementing the growth medium for the *A. laidlawii* with lipids of varying fatty acyl chain length.

The predictions were based on a theoretical analysis by Wilkins et al. [7] who showed that for lipid vesicles, the diffuse maxima observed in the small angle X-ray diffraction pattern are approximately related to the separation of the phospholipid head groups and submultiples of this distance [7]. The small angle scattering from *A. laidlawii* behaves in this fashion, the position of scattering maxima corresponding to the center-to-center spacing of phospholipid head groups in these membranes [6]. However, the relationship between X-ray scattering data from membranes and lamellar spacings in those membranes, is not always so straightforward. Theoretical analyses have shown that the simple set of diffuse X-ray diffraction maxima observed in scattering profiles from bilayers is extensively modulated in the presence of membrane proteins [8], and the positions of scattering maxima do not correspond to a bilayer thickness. Such effects were demonstrated experimentally by Blaurock [9] using lipid-cytochrome *c* vesicles. Recently it has been shown theoretically that the analysis of the scattering from lipid vesicles is also affected by the distribution of the size of the spherical particles [10] although in general this effect is relatively small. One purpose of these studies was to compare the small angle X-ray scattering of *E. coli* membranes with results of scattering from bilayers of *E. coli* lipids. Another purpose was to compare the small angle X-ray regions resulting from intact inner and outer membranes.

Purified forms of the cytoplasmic and outer membranes from *E. coli* can be obtained by centrifugation in sucrose gradients. Both membrane fractions are obtained in the form of single-walled vesicles as can be seen in electron micrographs of negatively stained specimens [11,12]. Although the glycerophosphatide compositions of the two membranes are similar [13], outer mem-

brane contains lipopolysaccharide as well as glycerophosphatide and protein. In addition, completely different protein compositions are associated with the two structures. The cytoplasmic membrane contains a number of transport systems and enzymes such as succinate dehydrogenase and NADH dehydrogenase as well as proteins such as cytochromes *a* and *b* [11,12]. The only known enzyme system in outer membrane is a phospholipase/lysophospholipase [13,14]. Most of the outer membrane is murein-lipoprotein, the most abundant protein in the cell [15]. These striking differences in the lipids and proteins found with the two membranes suggest that differences in the small angle X-ray diffraction profiles should be observable.

Materials and Methods

Materials. NADH, phenazine methosulfate, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), Brij-58, bovine γ-globulin, dipalmitoyl L-α-phosphatidylcholine, and all amino acids were purchased from Sigma Chemical Co., St. Louis, MO. Egg white lysozyme (salt-free) was purchased from Worthington Biochemical Corp., Freehold, NJ; palmitic acid was purchased from Nu-Chek Prep., Austin, MN, and succinic acid was purchased from Mallinckrodt Chemical, St. Louis, MO.

E. coli total lipids were extracted from frozen whole cells by the method of Bligh and Dyer [16].

Strains and culture conditions. All studies in this report utilized *E. coli* strain L-51, a *FadE* derivative of strain AB1623 which is defective in β-oxidation of fatty acids [17]. It was kindly provided by Dr. David F. Silbert. Cells were grown overnight in a flask at 37°C and innoculated to a visible turbidity in 50 l of medium 63 [18] supplemented with 0.4% glycerol, 1 μg/ml thiamine, 5 mM glutamate, 1 μg/ml yeast extract (Difco), 1 mM potassium acetate, 1.25 mM L-isoleucine, 0.4 mM L-leucine, 1.0 mM L-valine. Cells were grown under forced aeration with stirring at 37°C. Growth was monitored by measuring the absorbance of the culture at 600 nm. At an absorbance of 0.75—0.95, the culture was chilled to 20°C and harvested by continuous flow centrifugation.

Membrane preparation. Three somewhat different methods were used to purify inner and outer *E. coli* membranes. The first method was described by Osborn and coworkers [13]. In this procedure, freshly harvested, washed cells are converted to spheroplasts by lysozyme digestion in the presence of 1.0 mM EDTA, and the spheroplasts are ruptured by sonication of 30-ml aliquots for 45 s at a power setting of 7 or 8 on a Branson model S75 sonifier. Membranes are separated by centrifugation through a sucrose step gradient. Two bands of light membranes, L_1 and L_2, are combined and used as a source of inner membrane (referred to as L in this report). Band H was also collected as a source of outer membrane.

The second procedure was essentially that described by Yamamoto et al. [12] and involves spheroplast generation with lysozyme-EDTA at a 10-fold higher cell concentration than that employed by Osborn et al. [13]. Spheroplasts were ruptured in a French pressure cell, again at a high concentration. Membranes were repeatedly washed and collected by centrifugation at 78 000 × *g* and separated by centrifugation through 44% sucrose. Three fractions were

obtained, a hard pellet, a soft pellet, and a band, the first and third of which are putatively identified with fractions H and L of the Osborn procedure. Resuspension and centrifugation of fraction H in a 52%/54% sucrose step gradient [12] resulted in no further purification. In addition to the H and L fractions, this procedure also produced a third fraction, L′, which will not sediment from the ruptured spheroplasts at 78 000 × *g*. This fraction, designated by the term extra-light, was recovered by centrifugation at 250 000 × *g* and purified separately.

The third procedure was designed to avoid the production of the extra-light fraction, L′. The method of Yamamoto et al. [12] was followed except that spheroplasts were diluted and ruptures by sonication as in the procedure of Osborn et al. [13].

Whereever necessary, those fractions that were suspended in sucrose solutions were collected by centrifugation for 1 h at 250 000 × *g*. Heavy membrane pellets from procedures 2 and 3 were washed free of loosely pelleted material, resuspended in the growth buffer [18] and pelleted by centrifugation for 1 h at 250 000 × *g*. All membrane pellets were resuspended in the growth buffer and stored at 4°C or frozen and stored at −20°C. One preparation of membrane vesicles was studied by electron microscopy after staining with uranyl acetate; it appeared to contain vesicles similar in size to those obtained by other workers [11].

Assays. NADH oxidase was measured as described by Osborn et al. [13]. Succinate dehydrogenase activity was measured in an assay solution containing 60 mM potassium phosphate, pH 7.5, 10 mM NaCN, 30 μg MTT, 10 μg phenazine methosulfate, and 25 mM succinate in a volume of 1.0 ml. The reaction was started by addition of protein and followed at 25°C at a wavelength of 546 nm. The millimolar extinction coefficient of reduced MTT dye at this wavelength is 21, as determined from the data of Cartwright and Henning [19].

Protein concentrations were determined with the Bio-Rad dye binding assay [20] in a volume of 1.0 ml. Absorbance was read at 595 nm and calibrated with a bovine γ-globulin standard. None of the reagents present in the protein samples interfered with this assay. However, phospholipid contributed to the total absorbance. This contribution was eliminated by heating all samples together with 50 μl 0.1 M NaOH at 90°C for 15 min, then neutralizing with HCl prior to addition of the chromogenic reagent.

X-ray diffraction apparatus. The small angle X-ray camera was used with an Elliott GX-6 rotating anode generator operating at 35 kV, 17 mA, and using a copper target and a 100 μm focussing cup. CuK_α radiation ($\lambda = 1.5418$ Å) was selected with nickel foil and focussed with a double Franks mirror camera in an asymmetric arrangement, i.e. the source to mirror distance was different from that of the mirror to film. The source to sample distance was 285 mm and sample to film distances of 140—200 mm were employed. Accurate estimates of the camera magnification were obtained by recording the 9.087 Å X-ray reflection from a powdered basic beryllium acetate standard [21] placed at the sample position. The sample holder was an aluminum post mounted in a hollow teflon block (B in Fig. 1). The temperature of the sample was maintained to within ±0.5°C during the course of each exposure by pumping fluid from a

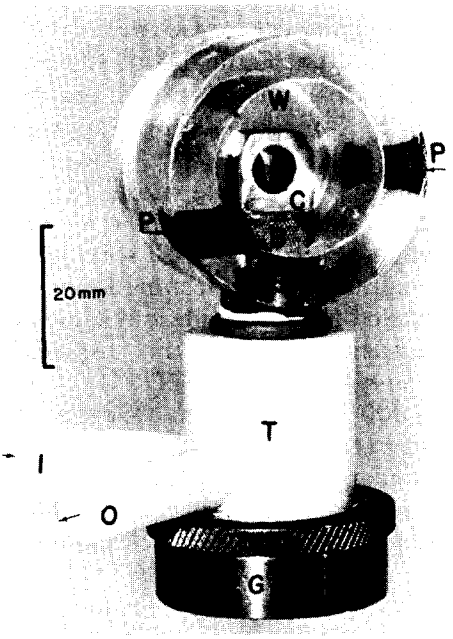

Fig. 1. Specimen holder for small angle X-ray scattering of membrane specimens. The specimen holder is connected to an X-Y translation via the base, G, having standard goniometer base threads. A hollow teflon block I has inlet and outlet ports (I, 0) for the circulation of constant temperature liquid. The specimen holder can hold a variety of types of aluminum posts. The one that is shown labelled B, extends into the liquid contained in T. This type is used to hold the small disc-like sample holders designed by Stroud and coworkers (personal communication) and used for high-speed pelleting of small quantities of membrane. Temperature can be monitored through either of the ports labelled P in the plastic drum used to maintain humidity around the specimen. The drum has thin mylar windows (0.0064 mm) labelled W and a tight seal to the aluminum post is made by a greased rubber gasket, S. For samples contained in sealed quartz capillaries, the thermostatting post has a simple pointed flat top on which the sealed capillary rests. The authors are grateful to E. Puronen for both the design and construction of the specimen holder.

circulating water bath through the teflon blcok (T in Fig. 1). The entire sample holder shown in Fig. 1 together with a small amount of buffer to maintain sample humidity was enclosed in a plexiglass drum with mylar windows (W in Fig. 1). Sample temperature was measured with a thermo-couple inserted through access ports (P in Fig. 1) in the wall of the drum.

Sample preparations and X-ray data collection. Aliquots of membrane suspensions (1—5 mg of protein) were diluted with the growth buffer and centrifuged at 100 000 × g for 1—2 h in an SW41 swinging bucket rotor. The membrane pellet was collected in a small (1 mm thick) disc-shaped device designed by Stroud and coworkers [22] (C in Fig. 1) for use with acetylcholine receptor membrane specimens. The disc plus an associated cone-shaped holder may be inserted into the centrifuge tube and then easily disassembled after a run. The disc having 0.0064 mm thick mylar windows can then be transferred to the aluminum sample holder in the X-ray camera without disturbing the membrane pellet. 1 or 2 ml of the growth buffer were also placed in the X-ray sample chamber to maintain humidity. The wet pellets contained approx. 25% (w/w) of membrane as determined by drying in vacuo over phosphorus pentoxide.

The X-ray data was collected on GAF No-screen and Ilford G Industrial X-ray film for exposure times ranging from 18 to 40 h. Estimates of the background scatter from the growth buffer used in suspending the membrane fractions were obtained by recording the small angle X-ray data with the appropriate blank.

Lipid vesicle preparation. Lipids dissolved in redistilled chloroform (20 mg/ml) were evaporated onto the walls of a conical centrifuge tube under a stream of dry nitrogen. The lipids were then suspended in 0.145 M KCl solution by heating to 50°C aided by sonication in a small ultrasonic cleaning bath. The turbid suspension was transferred to a thin-walled glass ampoule and sonicated while nitrogen was bubbled through the suspension. When the suspension appeared transparent, it was removed and centrifuged at 70 000 × g for 1 h. The supernatant was then centrifuged at 480 000 × g for 24 h to collect a lipid pellet for analysis. The lipid pellet was transferred with the aid of slow speed centrifugation (1000 rev./min) to a quartz capillary for the diffraction experiment. The temperature of the lipid samples was maintained at 9°C through an aluminum support post similar to that used for the membrane samples (B in Fig. 1).

Data processing. Absorbances of the small angle X-ray films were measured using an Optronics P-1000 drum scanning densitometer controlled by a Digital Equipment Corp. PDP 11/34 computer. Data were collected in selected sectors of the film and circularly averaged at 50-μm radial intervals. We are grateful to Mr. R. Wrenn and Dr. D. Ohlendorf for their contributions to the circularly averaging programs. Data from individual sectors were inspected for proper centering and averaged. Circularly averaged data are corrected for background and for geometrical and Lorentz factors as follows. To determine the appropriate background, an exposure was made of the background scatter using the

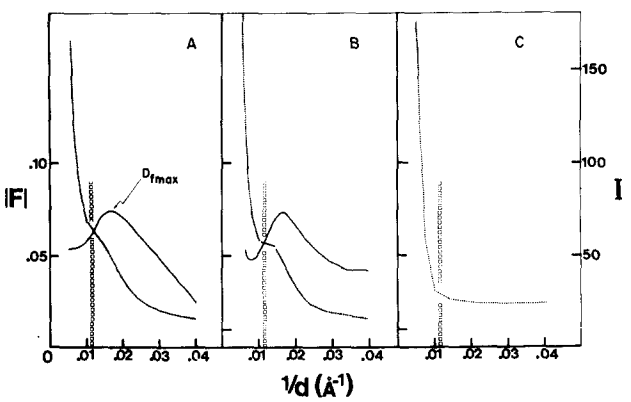

Fig. 2. Small angle X-ray diffraction profiles of purified membrane fractions. The profiles have been circularly averaged over segments of the film. The results shown in A are from the inner membrane, those in B from the outer membrane from *E. coli* obtained as described in the text. The curves in C are scattering from buffer in the sample cell. The dotted lines are raw but circularly averaged data. Units of intensity, I, shown on the right ordinate are proportional to the absorbance of the film. An absorbance of 2 is about 255 of the intensity units. The solid lines represent the fully corrected X-ray scattering amplitudes as described in the text using a constant background correction. The dashed column represents the reciprocal dimension of 1/87 Å^{-1}. As can be seen in the blank, C, the background is relatively constant beyond this point.

buffer instead of a membrane specimen. Typical results are shown in Fig. 2C. Note that beyond approx. 1/100—1/90 $Å^{-1}$, the background does not appear to change significantly. A first approximation to a background correction can therefore be made by selecting a value at a resolution where the scattering has become relatively constant and subtracting this from the small angle region. The corrected scattering amplitude, $|F| = I^{1/2} \sin \Theta$, where Θ is the scattering angle and I is an intensity which has been corrected for background.

Results

Membrane marker enzymes

The determination of the levels of marker enzymes can be used to evaluate the three methods of preparing purified inner and outer membranes which were employed in these studies. Since NADH oxidase and succinate dehydrogenase are localized on the inner membrane of *E. coli* [12,13], its membrane fractions could be identified by means of the specific activities of these enzymes. In addition to being indicators of purity, the specific activities of these marker enzymes may also be related to the structural integrity of the membrane.

Average values of the specific activities of NADH oxidase in the membrane fractions obtained in these two preparations are listed in Table I. The procedure of Osborn et al. [13] yielded a light membrane fraction which contained an average of 10.5 times as much NADH oxidase activity per mg protein as the heavy fraction. The specific activities in light and heavy membrane fractions from the procedure according to Yamamoto et al. [12] are both significantly lower than in the first procedure. However, as can be seen in Table I, the relative enrichment for NADH oxidase in the light fraction is increased slightly.

The isolation method of Yamamoto et al. [12] also yielded a membrane fraction, L′, which was purified in parallel with the L and H fractions. It consistently banded at a position corresponding to a slightly lower buoyant density than fraction L in sucrose density gradients. The specific activities of NADH oxidase (Table I) and succinate dehydrogenase (Table II) in this extra-light fraction are comparable in magnitude to the corresponding specific activities in the

TABLE I

SPECIFIC ACTIVITIES OF NADH OXIDASE IN MEMBRANES

Membrane preparations and assays are described in Materials and Methods. All specific activities are in μmol NADH oxidized/min per mg protein. Figures in parentheses are standard devations. The abbreviations L and H represent light and heavy membranes as described in Materials and Methods. L′ is the extra-light fraction which would not sediment at 78 000 × *g*. Procedure 1 was done twice and procedure 2 was done five times. The L/H ratio was calculated by averaging the ratios of the specific activities of fraction L to those of fraction H from the same preparations. In the case of procedure 2, in one instance, reliable data for both L and H could not be obtained in the same preparation, and L/H was not computed.

Procedures	Membrane fraction			Mean relative specific activities (L/H)
	L	L′	H	
1 (Osborn et al. [13])	1.34 (± 0.91)	—	0.16 (± 0.08)	10.5
2 (Yamamoto et al. [12])	0.172 (± 0.15)	0.175 (± 0.18)	0.024 (± 0.02)	15.1

TABLE II

COMPARISON OF PROCEDURES FOR RUPTURING SPHEROPLASTS FROM *E. COLI*

All the samples below were derived from a single spheroplast preparation. The enzyme assays are described in Materials and Methods. Succinate dehydrogenase specific activities are expressed in μmol MTT reduced/min per mg protein. Units of NADH oxidase activity are the same as in Table I. The ratios of the specific activities of fractions L and H are also given as in Table I.

Procedure	Specific activities							
	NADH oxidase				Succinate dehydrogenase			
	L	L′	H	L/H	L	L′	H	L/H
French press	0.069	0.088	0.008	8.6	0.069	0.081	0.020	3.4
Sonication	0.176	—	0.002	88	0.188	—	0.011	17

light fraction, suggesting it is also derived from the inner membrane.

Membrane fractions were also prepared by a third method which, like the first, avoids generation of the extra-light fraction L′. This method is derived from the method of Yamamoto et al. [12], and differs only in that it employs sonication in dilute suspension to disrupt spheroplasts. To check the effect of sonication, one spheroplast preparation was divided into two parts, using one for disruption by sonication, the other by the French press. The specific activities of the enzyme markers were then determined and the results are shown in Table II. The specific activities and relative enrichments of both marker enzymes in inner membranes prepared from sonicated spheroplasts were significantly higher than in membrane fractions prepared after cell disruption in the French pressure cell. Thus from these enzyme studies, it appears that sonication is the best method for cell disruption; but both types of preparations were studied by X-ray diffraction analysis.

X-ray scattering data

The small angle X-ray studies are divided into three parts which are those resulting from: (1) inner or cytoplasmic membrane, (2) the outer membrane, (3) sonicated dispersions of cellular lipids. The X-ray data from the intact purified membranes usually consisted of one diffuse principal maximum except as indicated in the next section. Typical results are given in Figs. 2a and 2b containing the circularly averaged diffraction curves for inner and outer membranes, respectively.

The shapes of the X-ray scattering profiles for both membrane forms are quite similar. As already noted, each is characterized by a single maximum. For simplicity, the position of this maximum will be referred to by its corresponding real space coordinate, which shall be called $D_{f\max}$. Because of the relatively large correction used to obtain the curves and the width of their maxima, values of $D_{f\max}$ for these two membrane forms are the same within experimental error. No higher order fluctuations in the diffraction pattern were observed for either form of the intact purified membranes, except for that from certain preparations of inner membranes. In that case, the small angle X-ray diffraction profile under some circumstances appeared to contain two

relatively close peaks. These maxima were not simple multiples of the peak occurring at the smallest angle. In fact, the absence of higher resolution features in the small angle region is a factor which by itself suggests that scattering is not dominated by a lipid bilayer [7,8], and one is cautioned not to interpret D_{fmax} values in terms of structural dimensions.

Inner membranes from E. coli

In examining purified forms of cellular membranes, perhaps the single most troublesome factor is the question of membrane stability during the manipulations used in the purification procedure and during storage, when the latter is necessary. To test the effects of some of these factors, X-ray diffraction profiles of inner membranes isolated after disruption by a French press were compared with similar fractions purified after disruption by sonication. To avoid repetitious presentation of X-ray scattering profiles, the data is reported simply by the position of the first diffuse maxima, D_{fmax} and Table III contains values for the inner membranes after spheroplast disruption by both methods. As can be seen from this data, inner membranes isolated after spheroplast disruption with the French press give values ranging from 61 to 64 Å. The average D_{fmax} for the inner membrane fraction of 62.4 Å (σ = 1.2 Å) is significantly different from the extra-light fraction (L') which has a value of 58.4 Å.

The X-ray diffraction films from the inner membrane fractions for two preparations appeared to have two relatively close maxima and the circularly averaged line profiles after densitometry confirmed this appearance, as shown in Fig. 3. The two peaks from the inner membrane fraction from preparation 1 as seen in the curve labelled b in Fig. 3 corresponded to 1/63.7 $Å^{-1}$ and 1/54.8 $Å^{-1}$. The corresponding form of the inner membrane fraction stored without freezing (Fig. 3a) clearly has a maximum with a visible shoulder at the same reciprocal space coordinates as curve b. The simplest explanation of these results is that two forms of membrane are present. Since the scattering maxi-

TABLE III

SMALL ANGLE X-RAY MAXIMA FROM L AND L' MEMBRANES OF *E. COLI*

D_{fmax} is the reciprocal position of the diffuse maximum in the small angle X-ray diffraction curves; it is described more fully in the text. The term "frozen" means the membrane fraction was stored at −20°C for approx. 5 days. Unfrozen fractions were kept at 4°C for periods ranging from 2 to 5 days.

Preparation	D_{fmax} (Å)		
	Inner membranes (L)		Extra-light membranes (L')
	Frozen	Unfrozen	Frozen
Spheroplasts-french press			
1	63.7 54.8 —	64.1 54.0	58.4 4.18
2	61.2 4.26	— —	58.4
3	60.7 —	— —	
Spheroplasts-sonication			
4		59.2 — 4.26	
5	68.8 57.5 4.15		

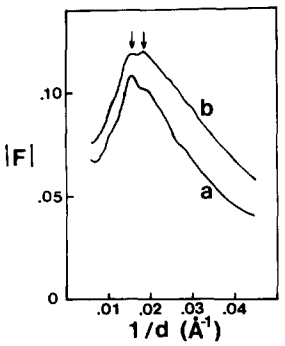

Fig. 3. Small angle X-ray diffraction profiles from purified inner membranes. The scattering amplitudes are plotted as a function of the reciprocal space coordinate. The line profiles are obtained after circularly averaging and correcting the data as described in the text. The curve labelled a is from purified inner membrane from *E. coli* stored at 4° C for about 2 days. The X-ray profile b was obtained from inner membrane which had been frozen at —20° C for approx. 4 days. Arrows mark the two diffuse maxima, which were also visible on the film.

mum occurring at about 1/64 $Å^{-1}$ is present in all preparations, the one occurring at 1/54 $Å^{-1}$ can be labelled transitory and would presumably be a form of inner membrane. Another equally plausible explanation for the additional peak in the small angle X-ray diffraction curve could be related to the loss or denaturation of peripheral membrane proteins. As noted in Introduction, the presence of peripheral proteins on bilayer structures modulates the scattering curve producing additional ripples in the very small angle region [8]. Thus, the slow and irreversible loss or denaturation of such proteins might also produce the variations given in Fig. 3 and Table III.

Inner membranes prepared and stored by all methods described here also exhibit weak, but sharp diffraction at 1/4.15—1/4.26 $Å^{-1}$, corresponding to the acyl chain packing distances observed in hydrocarbons [23] and phospholipids [24] below their melting temperature. The presence of this higher angle Bragg-like reflection is usually taken to indicate the presence of a phospholipid bilayer in these membranes.

Outer membranes from E. coli

Outer membranes from *E. coli* are characterized by diffuse profiles with maxima from 1/59.1 to 1/61.8 $Å^{-1}$. Values for D_{fmax} from four different preparations were determined. Like the cytoplasmic membranes, the small angle X-ray profiles are similar for outer membranes prepared using the French press and by sonication. Average values for D_{fmax} are 60.0 Å (σ = 1.2) and 60.4 Å (σ = .35), respectively. Furthermore, the diffuse maxima are insensitive to storage conditions since one preparation produced identical results before and after freezing.

In general, wide angle diffraction from these wet outer membrane fractions was weak. However, one sample displayed a sharp diffraction maximum at 1/4.25 $Å^{-1}$, consistent with the hexagonal packing of phospholipid hydrocarbon chains in the gel phase [24]. It should be noted that the small angle X-ray camera used to obtain the X-ray data is not well suited for obtaining the wide angle data of the X-ray diffraction pattern.

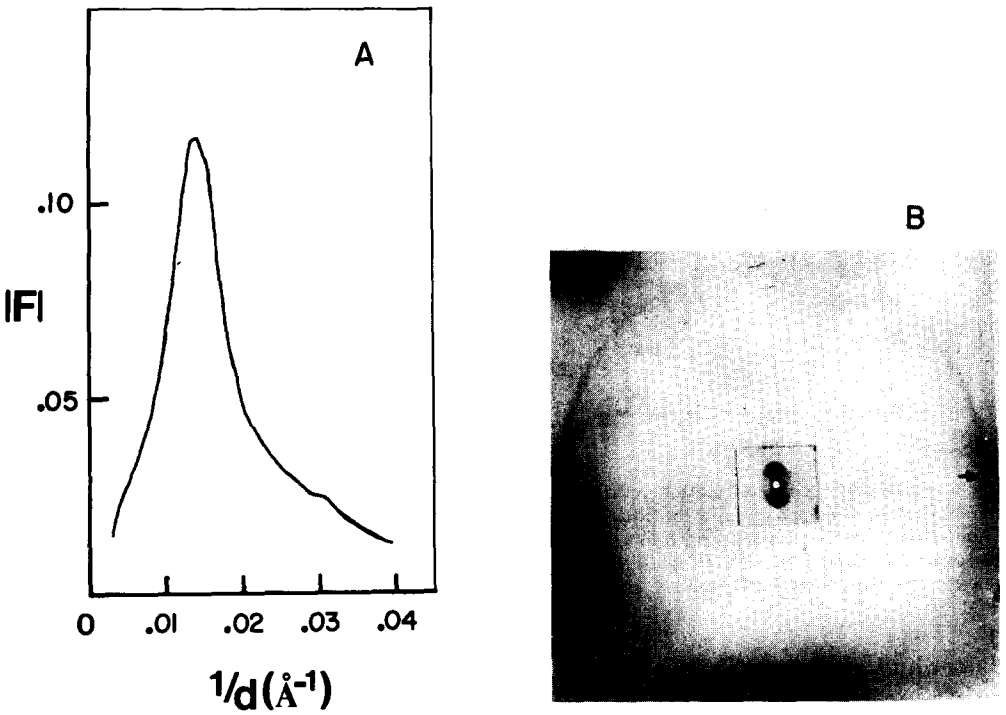

Fig. 4. X-ray data from partially dried outer membrane. The panel on the left contains a circularly averaged profile of the small angle region (60° sector along the meridian) of an X-ray diffraction pattern from purified and partially dried outer membrane. The X-ray diffraction photograph on the right is a composite from two films. The outer region (magnification = 144 mm) shows the wide angle X-ray data including the 4.2 Å acyl chain packing reflection which is partially oriented. This photograph was printed using a Kodalith Ortho internegative to enhance this weak reflection. Dodging by hand and using the original as a filter during printing minimized uneven exposure within the print. The central insert was printed from a shorter, more magnified X-ray exposure and shows a single, partially oriented X-ray maximum at about 1/73 $Å^{-1}$.

Because of the unusual components of the outer membrane, an experiment aimed at determining the nature of the underlying membrane structure was carried out. Partly dried specimens of outer membrane containing about 45% water produced partially oriented diffraction photographs with a small angle maximum in the direction of the meridian and a sharp high angle reflection at 1/4.19 $Å^{-1}$ with maximum intensity along the equator (Fig. 4). The small angle region shown in both Figs. 4A and 4B contains a single peak occurring at 1/73 $Å^{-1}$ with no other higher order fringes. The relative orientation of the high and low angle X-ray data is in agreement with bilayer models proposed for this membrane system [5,25].

Lipids

By way of comparison, the small angle X-ray data from total lipid extracts of *E. coli* were obtained and the results are shown by the solid line in Fig. 5. The average fatty acid chain length of phospholipids from strain L51 of *E. coli* grown at 37°C is 16.5 carbon atoms [26]. The dotted line in Fig. 5 also shows the small angle X-ray results from vesicles of dipalmitoyl phosphatidylcholine,

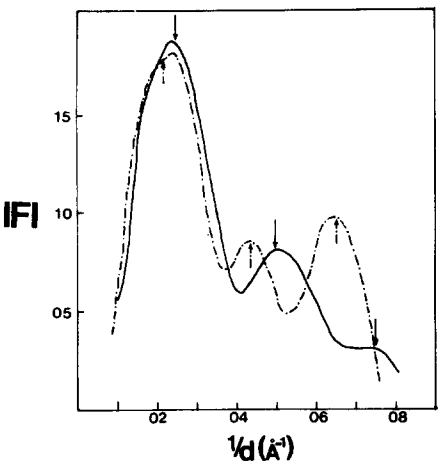

Fig. 5. Small angle X-ray diffraction profiles from *E. coli* lipid dispersions. The X-ray profiles have been obtained after circular averaging and the usual correction. The solid curve is from the total lipid extract from *E. coli* after reconstituting into vesicles. The dashed line represents similar data from dipalmitoyl phosphatidylcholine. The arrows mark D_{fmax} and submultiples of 46 Å for dipalmitoyl phosphatidylcholine and 42 Å for the total lipids of *E. coli*.

data which has already been described by other workers [6,27,28].

Higher order maxima are clearly visible in both X-ray profiles. Those from the dipalmitoyl phosphatidylcholine roughly correspond to a head group center to center distance of 46 Å [7]. The scattering maxima from a lamellar lipid phase re-formed from total lipid extracts of *E. coli* corresponds to a separation of about 42 Å between the centers of the head groups. The X-ray diffraction patterns from the dispersions of lipids from *E. coli* are more similar to the membrane results from *A. laidlawii* [6]. They are, however, quite different from the X-ray data obtained from either of the purified forms of intact inner or outer membranes from *E. coli* as shown in the previous sections.

Discussion

At the start of this investigation it was hoped that if X-ray data similar to that obtained with *A. laidlawii* membranes could be obtained from both the inner and outer membranes of *E. coli*, a crude comparison of their one-dimensional electron density profiles could be made. The X-ray data presented in the previous section show that such comparisons are not feasible. Other workers have found similar results [29]. The single diffraction peak in the small angle X-ray profile points to the fact that the diffraction pattern is modulated by the presence of relatively large amounts of protein or polysaccharide in both the inner and outer membranes. The positioning of such protein in terms of electron density profiles would be ambiguous especially because of the paucity of higher order maxima [8].

The similarities between the X-ray diffraction patterns from the inner or cytoplasmic and outer membranes from *E. coli* suggest that they may be qualitatively similar in structure. This similarity may be related to the fact that the lipid to protein ratio is about the same for both membranes [5,12], usually

approx. 0.7—1.0. The principal difference between the two membranes is related to the types of protein and lipid which are present. As stated earlier, the major protein in the outer membrane is the murein lipoprotein while about one-half of the outer membrane lipid is lipopolysaccharide [25]. The lipid from inner membrane from *E. coli* is almost totally phospholipid [12] and the inner membrane contains a variety of enzymes and transport proteins.

X-ray data from the inner membrane, although generally reproducible from one preparation to another, do exhibit some transitory changes in the small angle X-ray profiles. Because of the difficulties in obtaining large amounts of purified fractions and because of the weak nature of the diffraction patterns, it has not been possible to determine the nature of the transitory differences. It is suggested that the X-ray differences may be due to the loss of peripheral proteins.

In the case of the outer membrane, supporting evidence for an underlying bilayer structure is provided by the results from the partially dried specimen. The hydrocarbon reflection used to characterize phase transitions is clearly partially oriented and the small angle region also displays some orientation at right angles to the 4.15 Å reflection. The lack of higher order reflections in the small angle region of the X-ray diffraction pattern makes it impossible to use this region of reciprocal space to confirm an underlying lamellar arrangement.

The striking differences between the X-ray results from the reconstituted lipid vesicles and the purified membrane fractions can only be due to the absence of protein in the former case. In retrospect, since protein comprises over 50% of either membrane, the lack of scattering attributable to the juxtaposed electron dense head groups in a lipid bilayer region does not seem unusual. The fringes observed with lipid mainly in a bilayer arrangement are modulated by increasing the electron density in the hydrocarbon region, as occurs for integral membrane proteins. The same effect will occur by placing peripheral proteins or polysaccharides near the lipid head group regions. Current models for membranes suggest both locations for membrane proteins. In light of the results shown in the earlier section, a comparison of the distribution of lipid and protein and/or lipopolysaccharide in the inner and outer membranes from *E. coli* is not feasible. Small angle X-ray diffraction studies appear to be far better suited to purified proteins and reconstituted lipid systems than to the complex underlying structure of a cellular membrane.

Acknowledgements

We are grateful to Mr. R. Wrenn and Dr. D. Ohlendorf for their many contributions to the circularly averaging computing program used for densitometry of the X-ray diffraction films. We are also grateful to Drs. D. Silbert and J. Baldassare for bacterial cultures and frequent and helpful discussions of topics contained in this work, and to Tom Meininger for occasional technical assistance. The studies were supported by a grant from the N.I.H., GM 22595.

References

1 Silbert, D.F. (1975) Annu. Rev. Biochem. 44, 315—339
2 Shechter, E., Gulik-Krzywicki, T. and Kaback, H.R. (1972) Biochim. Biophys. Acta 274, 466—477

3 Shechter, E., Letellier, L. and Gulik-Krzywicki, T. (1974) Eur. J. Biochem. 49, 61—76
4 Linden, C.D., Blasie, J.K. and Fox, C.F. (1977) Biochemistry 16, 1621—1625
5 Overath, P., Brenner, M., Gulik-Krzywicki, T., Shechter, E. and Letellier, L. (1975) Biochim. Biophys. Acta 389, 358—369
6 Engelman, D.M. (1971) J. Mol. Biol. 58, 153—165
7 Wilkins, M.H.F., Blaurock, A.E. and Engelman, D.M. (1971) Nature 230, 72—76
8 Blaurock, A.E. (1973) Biophys. J. 13, 281—289
9 Blaurock, A.E. (1973) Biophys. J. 13, 290—298
10 Moody, M.F. (1975) Acta Cryst. A31, 8—15
11 Mizushima, S. and Yamada, H. (1975) Biochim. Biophys. Acta 375, 44—53
12 Yamamoto, I., Anraku, Y. and Hirosawa, K. (1975) J. Biochem. Tokyo 77, 705—718
13 Osborn, M.J., Gander, J.E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962—3972
14 Bell, R.M., Mavis, R.D., Osborn, M.J. and Vagelos, P.R. (1971) Biochim. Biophys. Acta 249, 628—635
15 DeMartini, M., Inyoue, S. and Inouye, M. (1976) J. Bacteriol. 127, 564—571
16 Bligh, E.G. and Dyer, D.T. (1960) Can. J. Biochem. Physiol. 37, 911
17 Davis, M.T. and Silbert, D.F. (1974) Biochim. Biophys. Acta 373, 224—241
18 Pardee, A.B., Jacob, F. and Monod, J. (1959) J. Mol. Biol. 1, 165—178
19 Cartwright, J.B. and Henning, U. (1970) J. Bacteriol. 102, 722—728
20 Bradford, M.M. (1976) Anal. Biochem. 72, 248—254
21 Tulinsky, A., Worthington, C.R. and Pegnataro, E. (1957) Acta Cryst. 12, 623
22 Ross, M.J., Klymkowsky, M.W., Agard, D.A. and Stroud, R.M. (1977) J. Mol. Biol. 116, 635—659
23 Vand, V. (1953) Acta Cryst. 6, 797
24 Luzzati, V. (1968) in Biological Membranes (Chapman, D., ed.), pp. 71—123, Academic Press, New York
25 Emmerling, G., Henning, U. and Gulik-Krzywicki, T. (1977) Eur. J. Biochem. 78, 503—509
26 Baldassare, J.J., Rhinehart, K.B. and Silbert, D.F. (1976) Biochemistry 15, 2986—2995
27 Lesslauer, W., Cain, J.E. and Blasie, J.K. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1499
28 Zaccai, G., Blasie, J.K. and Schoenborn, B.P. (1975) Proc. Natl. Acad. Sci. U.S. 72, 376—380
29 Linden, C.D., Blasie, J.K. and Fox, C.F. (1977) Biochemistry 16, 1621—1625.