Neutron Scattering Analysis of Bacterial Lipopolysaccharide Phase Structure

CHANGES AT HIGH pH*

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The aggregate structure of lipopolysaccharide isolated from an Re strain of Escherichia coli was examined at different pH values using small angle neutron scattering. At pH values of 6 and 7.4, angle-averaged scattering of the sodium salt of this isolate was consistent with randomly coiled tubular micelles approximately 100 Å in diameter. At pH 9.1, however, Kratky analysis of the scattering data was distinctly different and consistent with pairing of uniform tubular micelle sections of length 1440 and 110 Å in diameter. Contrast variation measurements of the micelles yielded an average micellar weight of the sample at pH 9.1 of approximately 1.11 × 10^7 daltons and suggested that the aggregates were tubular micelles of size and length similar to that derived from the scattering intensity data. Anisotropic scattering patterns of samples under shear indicated a rigidification of the micelles as the pH was increased to 9.1 and the temperature decreased from 25 to 10 °C. The rotational diffusion constants deduced from the observed shear anisotropy indicate that the structure at pH 9.1 must have smallest and largest dimensions which differ by at least an order of magnitude, ruling out spherical or moderately ellipsoidal structures. Analysis of the shear rate needed to induce anisotropic scattering indicated that the stiffness length of the micelles at pH 9.1 was approximately 1000 Å and decreased at higher and lower pH values.

A variety of physical techniques has been used to measure the architecture and conformation of isolated LPS from smooth and rough strains of enteric bacteria (1–6). While some investigators suggest that under most conditions extracted LPS exists as bilayers (1, 5), others have indicated that, under certain conditions, LPS may form nonlamellar aggregates (2–4, 6). Recent studies have suggested that ReLPS may form inverted micellar structures (3, 7), and that LPS from smooth strains (8) as well as rough LPS under conditions of high pH (7) forms tubular micelles. To further characterize the pH-dependent changes in the architecture of LPS aggregates, the sodium salt of LPS from an Re mutant strain was analyzed using small angle neutron scattering. The results indicate that at neutral pH the aggregates exist as randomly coiled tubular micelles, but at pH 9.1 the micelles are paired. Analysis of the samples under shear indicates that the micelles stiffen as the pH is increased from 7.4 to 9.1 and the temperature is lowered below 25 °C. We propose that upon elevating the pH to 9.1 the randomly coiled micelles stiffen and loop back on themselves to form closed tubular micelles.

EXPERIMENTAL PROCEDURES

Lipopolysaccharide Preparation

Escherichia coli strain 12182 (an ReLPS producing strain, 9) was grown at 37 °C in nutrient broth, and the LPS was extracted using phenol/chloroform/petroleum ether as previously described (10). The sample was further purified by sedimenting the aggregate several times at 75,000 × g for 120 min and resuspending the sample in distilled water. The sodium salt of LPS (NaLPS) was obtained by dialyzing the extract against five changes of 10 mM sodium ethylenediaminetetraacetic acid, pH 7.0, at 4 °C, followed by extensive dialysis against distilled water. Elemental analysis of the sodium salt was carried out as described previously (10). The levels of the LPS were quantitated using the thiobarbituric acid assay for 3-keto-d-manno-octulosonic acid (11) and by the dry weight of lyophilized samples. The samples were shown to contain less than 1% protein (by weight) using the Lowry method (12). The levels of nucleic acid were determined to be less than 1% by measuring the absorption at 260 nm. For neutron-scattering studies, lyophilized samples were weighed and suspended at 10 mg/ml in either 50 mM sodium phosphate (pH 6.0 and 7.4) or sodium borate (pH 9.1 and 9.5) containing the appropriate level of D_2O. The LPS isolate was identical for all of the pH studies. D_2O was redistilled prior to its use in the buffers. All buffers were adjusted to the indicated pH using a combination glass electrode and pH values were not corrected for the presence of D_2O.

Neutron-scattering Studies

Static-scattering measurements were done using 1-mm path length cylindrical quartz cells and measured at 25 °C. For scattering of samples under shear, a concentric cylindrical, water-jacketed quartz cell with a total optical path of 1 mm was used (13). The exterior cylinder of the cell was motor driven, and the rate of rotation was monitored. At the maximum speed of 3000 rpm, a shear rate G = 18,500 s⁻¹ was obtained.

Neutron-scattering measurements were performed at an incident wavelength λ = 4.75 Å on the 30-m small angle neutron-scattering instrument of the National Center for Small Angle Scattering Research at Oak Ridge National Laboratory. Slits diameters of 12 mm at the sample and 25 mm at the source were used in conjunction with a sample-to-detector distance of 12 m. Data were corrected for sample transmission, cell and spectrometer background, solvent scattering, detector efficiency, and incoherent scattering from the sample, using well-established standard techniques (14). (The resulting statistical errors are marked as vertical bars on all intensity data presented.) Absolute intensity was calibrated using an irradiated aluminum scat-
tering standard (15). A constant systematic error in the absolute scale, estimated to be ±5%, may be present in all runs; statistical errors in the relative intensities are generally ±2%.

In the present study, the contrast of the sample was varied by the H2O/D2O ratios in the solvent. For other analyses of sample shape by static, angle-average scattering as well as asymmetric scattering under shear, the sample was suspended in 81% D2O since the transmission is higher in D2O than in pure H2O.

Guinier Analysis—The angle-averaged scattering data can be used to determine the radius of gyration of a particle. In the low k region, Guinier's law states: \( I(k) = I(0) \exp(-k^2R^2/3) \) where \( I(0) \) is the forward scatter, \( k = (4\pi/\lambda) \sin \theta \), and \( R \) is the radius of gyration. \( I(0) \) and \( R \) were determined by a plot of \( \ln(I(k)) \) versus \( k^2 \). \( I(0) \) is related to the aggregate's particle weight and its partial specific volume.

Kratky Analysis—Data collected to large k values were interpreted by the method of Kratky and Pitz (16). This is a search method which assumes that the shape of the particle can be approximated by simple geometric objects. The scattering curve of \( k^2 I(k) \) as a function of k is then compared to different models and analyzed for the best fit. To test the static scattering data, particles of randomly oriented coils, uniform cylinders, and paired uniform cylinders were considered; the expected Kratky plot of scatter from such particles was calculated.

The shear data was resolved into scatter parallel, \( I(\parallel) \), and perpendicular, \( I(\perp) \), to the fluid flow, and tested against models of oriented cylinders (13). The calculations for the expected scatter from such models under shear are also given.

Rotational Diffusional Analysis of Samples under Shear—Broersma (17) has shown that for a cylinder of radius, a, and stiffness semi-length, \( \tau \), the diffusion coefficient, \( D \), would be related to length and diameter by the following relationship: \( D = 3k_BT/(\pi - t)/8\eta a^3 \), where \( a \) is the solvent viscosity, \( \tau = \log(2/a) \), and \( t = 1.57 - (70.28 - 1)/s \). We would expect the onset of anisotropy when \( G/D \approx 1 \). Thus, the shear rate corresponding to the onset of anisotropic scattering was used to estimate the stiffness length of the micelles. This is the average length of the aggregate without a bend. For these analyses, the diameters of the micelles were assumed to be between 100 and 160 Å, consistent with the values obtained in the static-scattering experiments and with the known monomer dimensions. The estimated values were then refined by comparison with detailed model calculations ("Results").

An important feature of this type of experiment is that the very existence of anisotropy in the scattering, under the mild shear conditions used, rules out entire classes of possible structural models. In the present case, any proposed model must lead to a rotational diffusion coefficient corresponding to the observed value ~2000 s-1, regardless of any other features. Structures with spherical symmetry are too short, and lamellar structures would not show anisotropic scattering in the particular shear geometry used. Either cylindrical or ellipsoidal shapes are possible, provided their axial ratios are of order 10:1; at this axial ratio, the two shapes are practically indistinguishable, and we have used cylinders for the model calculations.

RESULTS

Chemical Characterization of Isolated LPS—The chemical structure of the LPS from E. coli strain D21F2 has been analyzed by several investigators (18, 19) and is shown in Fig. 1. The level of monophosphate and pyrophosphate at the reducing end of the lipid A head group is variable and presumably depends on the growth conditions (20). Thus, analysis of the phosphate content of the NaLPS was carried out using plasma emission spectroscopy (10). Elemental analysis also indicated that the isolate was neutralized with Na+ and had only very low levels of contaminating multivalent metal cations. The thiobarbituric acid assay indicated that the sample contained greater than one 3-keto-D-manno-octalosonic acid unit. We assume that the sample contained two 3-keto-D-manno-octalosonic acid/LPS, but under the mild acid hydrolysis conditions used, incomplete release of the sugar resulted in incomplete detection of the residue. Since the sample was shown to be free of protein and nucleic acid contamination, we used the dry weight of lyophilized LPS to quantitate the amount of material.

Angle-averaged Static Scattering—The angle-averaged scattering patterns of the NaLPS at pH 6.0, 7.4, and 9.5 did not differ substantially (Fig. 2). Data at these three pH values yield linear \( \log I(k) \) versus \( \log k \) plots, with slopes close to the value (−2) characteristic of a random coil structure. None of the data shown in Fig. 2 yields a linear Guinier plot (\( \log I(k) \) versus \( k^2 \)), but estimates of the largest and smallest dimensions can be made from the asymptotic low and high k Guinier slopes. Except at pH 9.1, this yields characteristic small and large radii of gyration of order 47 and 280 Å, and the data are consistent with "flabby" tubular micelles in a loosely coiled configuration. At pH 9.1, however, the data differs qualitatively from that at lower or higher pH, with the existence of a k-range in which the slope is −1, indicative of very elongated particles.

The intensity of scatter at pH 6.0, 7.4, 9.1, and 9.5 was fitted to modeled data. The \( I(k) \) versus k and Kratky plots for different shaped particles were calculated. A random coiled particle would be expected to give a straight line Kratky plot; i.e. \( k^2 I(k) \) would be approximately constant, and this was found to be the case for the scattering data for the NaLPS at pH 6.0, 7.4, and 9.5. In contrast, at pH 9.1, the Kratky plot was unique and showed pronounced structure (Fig. 3). Anal-
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-2.0

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4.0

LOG K

FIG. 2. Angle-averaged scattering intensity, I(k), as a function of momentum transfer, k, for samples measured at 25 °C and at the indicated pH values. On the log-log scale used, a straight line of slope -2 is indicative of a random coil structure.

FIG. 3. Kratky plot of k²I(k) versus k for NaLPS at pH 9.1 at 25 °C. The dotted line indicates the expected results for scattering from paired cylinders 100 Å in diameter. Scattering from a random coil would give a constant value in this plot.

Anisotropic Scattering—Anisotropic scattering patterns of NaLPS at pH 7.5, 9.1, and 9.5 were taken as a function of applied shear, G (0 < G(s⁻¹) < 2 × 10⁴), in the temperature range of 10 to 25 °C. The anisotropy was more pronounced at the lower temperatures and was significantly pH dependent (Fig. 5). Analysis of the shear rate needed for the onset of anisotropy (13) indicated that the samples at pH 9.1 and 9.5 appeared to have persistence lengths of 800 to 1000 Å at temperatures of 10 to 15 °C (Table II), consistent with the lengths determined using the contrast variation data and intensity data. These lengths dropped significantly when the temperature was raised to 25 °C. Fig. 5 indicates the level of anisotropy in scattering at different shear rates. These results show that although the persistence length of aggregates at pH 9.1 are similar at 10 and 15 °C, the sample at the lower temperature was significantly more aligned. Analysis of the sample at pH 7.5 showed that there was little alignment even at 15 °C. Presumably, the stiffness length at pH 9.1 is maximal.

FIG. 4. The contrast variation of NaLPS at pH 9.1 measured at 25 °C. The forward scatter intensity of neutrons was measured at different D₂O/H₂O ratios. Error bars include estimated systematic errors in the absolute intensity scale.

FIG. 5. The anisotropy of scattering of neutrons by NaLPS in a shear field. Scatter from samples at pH 7.4 (half opened symbols) 9.1 (open symbols), and 9.5 (closed symbols) were measured at 10 (triangles), 15 (circles) and 25 °C (squares) at different shear rates, G. The axial ratios of the scattering contours were measured as shown in the insert.
TABLE II
Persistence length (Å) of tubular micelles determined by the onset of anisotropic scattering

| Temperature | pH  | Length of Micelles |
|-------------|-----|---------------------|
| °C          | 7.4 | 9.1                 |
| 10          | ND* | ~1000               |
| 15          | <700| ~1000               |
| 25          | ND  | <1000               |

* ND, not determined.

The lengths were determined by measuring the shear rate needed to align the samples as described under "Experimental Procedures." The results assumed the aggregates were micelles with a diameter of 100–160 Å, that the viscosity of the solution was 0.01 poise, and that T = 300 K.

**DISCUSSION**

The contrast variation results, the anisotropic-scattering patterns, and the Kratky plots of static and oriented samples are consistent with LPS at pH 9.1 aggregating as regular tubular micelles with lengths of 1200 Å or more. The volume of the micelles calculated from the contrast variation data is 1.37 × 10^6 Å³, while the model derived from the best fit of the Kratky plot of paired cylinders indicates a volume of 1.36 × 10^6 Å³. The smaller characteristic length at pH 9.1 determined from the Guinier analysis was approximately 40 Å and indicates that the micelles have diameters of about 80–100 Å, consistent with the diameter suggested by the model (110 Å). Such tubular micelles have been visualized in electron micrographs of LPS from smooth strains (8) as well as from rough strains in the Li salt form. As reported previously, the viscosity of the LPS solution at pH 9.1 was high and dropped at higher and lower pH values (7). Furthermore, the anisotropic-scattering data indicated that the stiffness of the micelles increased as the temperature dropped from 25 to 10 °C, due perhaps to an ordering of the acyl chains within the micelles. For the sample at pH 9.1, the static scattering intensity at 25 °C and the resolved anisotropic scattering at 10 °C was unique and suggested that the micelles were paired. We propose that this pairing is the result of the tubular micelles closing back on themselves, forming hairpin-like micelles with both ends closed. Such closed tubular micelles of LiLPS from an Ra strain have been seen in the electron microscope. The forces that stabilize the micellar form at pH 9.1 are thought to be the high head group charge repulsion and the steric hindrance of the hydrated head group region when the charge is high. Above pH 7.5, the second ionization of the two phosphates causes a dramatic increase in the negative charge of the lipid A head group. Charge repulsion and higher levels of bound water may induce an increase in head group spacing and, thus, in curvature of the aggregate. Since the LPS head group has a long and short axis (1), LPS may not readily pack into a spherical micelle; thus, the tubular micelle would be the stable form under these conditions. However, lipid at the end of a tubular micelle would pack as a sphere. If spherical packing is unstable at pH 9.1 due to the geometry of the molecules, ends of tubes would tend to coalesce, forming either longer tubes or circularly closed tubes.

At the lower pH values, 6.0 and 7.5, the scattering was consistent with floppy tubes of larger average diameter than at the higher pH. Such floppy tubes did not align in the shear cell, presumably due to the flexibility or lack of asymmetry in the aggregates. This type of irregular tube or ribbon has been visualized in electron micrographs of NaLPS from rough strains (8). As reported previously, the samples at neutral pH were cloudy compared to the clear samples at basic pH (7). This difference in light scattering also reflects differences in aggregate size.

When the pH of the sample was raised above 9.1, the angle-averaged scattering pattern was no longer consistent with paired tubes. The sample remained clear and still readily aligned in the shear cell. However, the viscosity of the sample at pH 9.5 was significantly lower than at pH 9.1. We propose that further increasing the charge on the LPS at pH values above 9.1 further increases the head group spacing within the lipid aggregates. This may allow for the formation of semi-spherical micelles at the ends of the tubes. If the driving force to form closed loops and longer tubes is lessened, the micelles may then become smaller and unpaired. This is what occurred at pH 9.5. The anisotropic-scattering data indicated that the length of the micelles at pH 9.5 was shorter than at 9.1 (Table II). The decrease in the length of the micelles may account for the decrease in viscosity. We reiterate that the mere observation of anisotropic scattering, in our experimental geometry and at the shear rates used, rules out lamellar structures and globular structures with axial ratios less than about 10:1, regardless of any specific model features.

In conclusion, our results indicate that LPS from an Re strain can adopt several different micellar forms in the basic pH range. These conformational changes appear to be driven by charge interactions within and between the head groups of the molecules. Similar charge group interactions are likely to occur between LPS molecules from other rough as well as smooth strains (7). The formation of nonlamellar aggregates...
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of LPS on the surface of the bacterial cell may also occur under specific conditions and may account for the release of LPS from bacterial cells.

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SUPPLEMENTARY MATERIAL TO "Neutron Scattering analysis of bacterial lipopolysaccharide phase structure. Changes at high pH."

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Calculations for Models

Analysis of the size of lipopolysaccharide molecules and aggregates based on small-angle neutron data.

The total neutron scattering amplitude $I$ of the molecule shown in Fig. 1 is equal to $I(1)(k)$, where $k$ is the vector of the incident neutron beam. The molecular mass $M$ of this molecule is 255.2 $\times$ 2 $\times$ 24, if $k$ is the vector density of spheres of molecule, each with an aggregation number of $n$, then an average concentration of lipopolysaccharide (LPS) used in these experiments is given by:

$$I(1)(k) = n^2 k^2 M$$

For aggregates of this molecule, the intensity of the scattering of a single particle, is (10):

$$I(0)(k) = k^2 M$$

where $k$ is the volume of the LPS, $k$ is the volume on each of the scattering amplitudes of the molecule. Substituting $k = 0$ and $k$ in this equation:

$$I(0)(k) = k^2 M$$

For $k = 0$:

$$I(0)(k) = k^2 M$$

A least-squares fit to the data of Fig. 4 yields $I(0)(k) = 0.19 + 0.11$, so that $k = 0.000$ and $M = 100$. For the slope of the $I(0)(k)$ vs. $k$ plot (Fig. 4) is $21.5 + 0.5$. The coefficients of $k$ in a plot of $I(0)(k)$ vs. $k$ can be calculated by letting the area under the curve be $a$ of the slope of equation $x$. The slope is the slope $k = 21.5$ of $I(0)(k) = 0.19 + 0.11$. Using our value of $k$, and comparing with the measured slope then gives an aggregation number $n = 450 + 50$.

The slope of the average $I(0)(k)$ is $21.5$. If the aggregate is assumed to be a uniform cylinder with radius of $a = 5 A$, then:

$$I(0)(k) = 21.5 \times 5$$

where $a$ is the length of the cylinder, from which $I(0)(k) = 100 + 10$. The mean density of these molecules and the aggregation of the aggregates would be:

$$k = 0.000 + 0.11 + 0.19$$

$$k = 0.000 + 0.11 + 0.19$$

Calculated neutron scattering intensities for specific particle shapes - random models assumed.

The formalism for neutron scattering is similar to that for light or X-ray scattering. In the absence of any simplifying features that allow us to obtain an analytical expression for the scattering from a given atom, we must resort to an approximation known as the shell model of scattering. The scattering from a uniform particle may be written as the Fourier transform

$$I(k) = \frac{1}{V} \int d^3 r \rho(r) \rho(0) e^{-i k \cdot r}$$

where $I(k)$ is the scattering amplitude per unit volume, and the integral is over the volume $V$ of the particle. For single spheres, both as cylinders, the scattering cross-section is given by:

$$I(k) = \pi a^2 h k$$

where $a$ is the radius of the sphere, and $h$ is the contrast between the particle and the medium. For two different spatial positions, they will have a joint spatial orientation $S$, and the scattering from a sphere due to a sphere is given by:

$$I(k) = \pi a^2 h k S$$

where $S$ is the number of segments (each of length $a$) in the chain.

For $a = 1$:

$$I(k) = \pi a^2 h k S$$

Thus, the average $S$ of $I(0)(k)$ would be:

$$S = \frac{1}{n}$$

where $n$ would give a straight line of slope zero.

**Tetragonal cylinders**

For randomly oriented cylinders of length $2a$, radius $a$, and scattering amplitude $S$, we expect the following intensity distribution of scattered neutrons to be given in equation 4.

$$I(k) = \frac{(2a)^2}{\pi} \int \frac{|S|}{(a^2 + k^2)^2} ds$$

where $S$ is the scattering cross-section (in barns) of the cylinder, and $\sigma$ is the total cross-section of the cylinder.

**Tetragonal arrays of cylinders**

For randomly oriented pairs of cylinders of length $2a$, radius $a$ and separated by a distance $d$, the scattering cross-section of the cylinders is given by equation 4. The scattering intensity of such a pair of empty cylinders is given by:

$$I(k) = \frac{(2a)^2}{\pi} \int \frac{|S|}{(a^2 + k^2)^2} ds$$

where $S$ is the scattering cross-section of the cylinder, and $|S|$ is the total cross-section of the cylinder.
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have to be averaged over all orientations ($\hat{P}_{\perp} = -\hat{P}_{\perp}$). Without loss of generality, we may take the long cylinder axes parallel to $\hat{a}$ and average over all (010) directions for $\hat{b}$ (in standard polar coordinates). We then

\[ I(x) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

where $\beta = \cos \beta + \sin \beta$, this reduces to:

\[ I(x) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

Since $\int_0^{\pi} \sin \theta \sin \phi \cos \phi \cos \theta \, d\theta = \pi \sin \phi \left( \cos \theta + \cos \theta \right)$, we finally obtain:

\[ I(x) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

Calculated neutron scattering densities for shear-aligned oriented cylinders.

We need only consider the case of paired cylinders, since the single cylinder result is then obtained as a special case. The form-factor for an oriented cylinder is

\[ F(\hat{a}, \hat{b}) = 2M \sin \phi \left( 1 - \cos \phi \right) \]

where $\phi$ is the angle between $\hat{a}$ and the cylinder axis.

We choose coordinates with the $\hat{a}$ axis in the direction of the shear gradient and the $\hat{b}$ axis in the direction of the spin, and let standard polar angles $\beta$ and $\theta$. Since the detector is in the plane, $\hat{z}$ lies in the $\hat{a}$-$\hat{b}$ plane. Then, if $\gamma$ is the angle between $\hat{a}$ and $\hat{b}$, the expression is obtained by changing $\gamma$.

We let $p(\hat{a}, \hat{b})$ be the probability that the pair of cylinders is oriented in the direction at spin. $\hat{a}$, where $p(\hat{a}, \hat{b})$ is the rotational diffusion coefficient. We can then use the Majorana-Field Form for this probability, assuming that each cylinder is unaffected by the other [13]. The main effect of pairing will correspond to an effective viscosity different from the actual viscosity.

Choosing the origin at the center of symmetry of the pair of cylinders, the form-factor is simply obtained from equation (4) with appropriate phase-factors:

\[ F(\hat{a}, \hat{b}) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

where $\hat{a}$ is the vector joining the centers of the cylinders. In the general case, the long cylinder axis is parallel to the $\hat{b}$ direction, and we must average over all $\hat{a}$ and $\hat{b}$ for a given $\beta$. Further, the pairs of cylinders can rotate about their own long cylinder axis, i.e., the rotation angle is $\gamma$. We assume this rotation is free and uncorrelated with $\phi$ and $\beta$, or assumption which will not be incorrect to detail, but which should not affect the main physics.

From trigonometry we obtain the following relationship:

\[ L \hat{a} = \hat{b} \cos \beta + \hat{b} \sin \beta \]

where $L$ is the case (case case case = case case) and $\beta$ is the case case.

The intensity at a given shear characterized by $\hat{b}$ and for a given $\beta$ is then

\[ I(x) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

Integration of equation 8 over $\phi$ can be performed analytically under the assumption that $\phi$ is uncorrelated with $\beta$ and $\theta$ to yield the intensity distribution function

\[ I(x) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

where $p(\phi, \beta) = \pi \sin \phi \left( \cos \theta + \cos \theta \right)$.

In the special case where $\theta = \beta$ and $\phi = 0$, then:

\[ F(\hat{a}, \hat{b}) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

and integration of equation 8 over $\phi$ can be evaluated analytically. Leaving a single numerical integration (see Ref. 13 for details).

In the special case where $\beta = 0$ (i.e., $\phi = 0$), then:

\[ I(x) = \frac{\alpha}{\beta} \int_0^{2\pi} \frac{d\phi}{\beta} \int_0^{\pi} \frac{d\theta}{\beta} \sin \theta \left( I(0) \cos \theta + I(\pi) \sin \theta \right) \]

and integration of equation 8 must be undertaken numerically over both $\beta$ and $\phi$. 