Effect of pH on Solubility and Ionic State of Lipopolysaccharide Obtained from the Deep Rough Mutant of *Escherichia coli*†

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ABSTRACT: The dissociation of the highly aggregated form of lipopolysaccharide (LPS) from Gram-negative bacteria to the monomeric (or soluble) form is though to be the initial step in the activation of responding cells (macrophages, B-cells, neutrophils, monocytes, and endothelial cells) by LPS. This process is presently not adequately understood. Using the equilibrium dialysis apparatus and a highly purified and well-characterized radiolabeled deep rough chemotype LPS ([^{14}C]ReLPS) from *Escherichia coli* D31m4, we have examined the effect of pH on its solubility (C_T) and ionic states in aqueous media. The solubility range of [^{14}C]ReLPS suspended in 50 mM Tris-HCl–100 mM KCl buffer (or 50 mM MES–100 mM KCl buffer at pH 6.5) was determined to be from (2.91 ± 0.01) × 10^{-8} to (4.55 ± 0.07) × 10^{-8} M over a pH range of 6.50–8.20, respectively. These experimental data satisfactorily fitted the curve generated by the solubility equation $C_T = S_0(1 + K_5/[H^+])/([H^+]/K_4' + 1)$, where S_0 is the concentration of the tetraanionic ReLPS, K_5 is the dissociation constant of the tetraanionic ReLPS in solution, and K_4' is the dissociation constant of the trianionic ReLPS at the surface of the solid particles in suspension. The increase in solubility of ReLPS with increase in pH from 7.00 to 8.20 is primarily caused by the formation of the pentaanionic form from the tetraanions. The pK_5 (primarily the second dissociation of the 1-phosphate) of ReLPS was determined to be 8.58 from experimental data. Theoretical arguments were presented to show that this value is higher than that for simple model compounds (monosaccharide monophosphates where pK = 6.1 for the second dissociation) because of electrostatic effects caused by the other phosphate and carboxylate groups of two 2-keto-3-deoxyoctonate (Kdo) moieties of ReLPS. Using the same theoretical arguments, pK_6 was calculated to be much higher, 10.8. In the absence of Kdo groups, as is the case of 1,4′-diphosphoryl lipid A, the same theoretical approach showed that the pK values of the second dissociations of the two phosphate groups are lower and are separated by smaller numbers, giving calculated pK values of 6.9 and 7.8. The presence of nearby Kdo units in ReLPS gives the molecule fewer negative charges on the phosphate groups compared to lipid A. This may contribute to better binding of ReLPS to the LPS receptors and may explain its higher biological activity when compared to lipid A. From these results, we can now provide the monomeric concentrations, pK values, ionic states, and charge distribution of a model, toxic LPS dissolved in aqueous media. Such information is necessary to understand the molecular basis for the biological activities of LPS.

Lipopolysaccharide (LPS)[1] is an amphipathic macromolecule found on the outer surface of the outer membrane of Gram-negative bacteria (Nikaido & Vaara, 1985). It consists of hydrophilic polysaccharide and lipophilic lipid A moieties (Rietschel et al., 1982). The isolated LPS has a wide range of immunological and pathophysiological activities (Nowotny, 1983), but most of these biological activities can be directly attributed to the lipid A region.

Since LPS is highly aggregated in aqueous medium, questions were raised as to the physical nature of the biologically active LPS unit. Is it an aggregate with a defined surface topography or a monomeric LPS exhibiting specific structural features in the lipid A region that are recognized by the biological system? If monomer, what are its solubility properties? Is the available monomer concentration adequate for activating the biological systems? The answers to these questions are basic to how we interpret the results of studies on the receptor-to-ligand interaction and the structure-to-function relationship of LPS or lipid A in the activation of responding cells (macrophages, B cells, neutrophils, monocytes, and endothelial cells). These questions have not been adequately resolved.

Two lines of evidence favor monomeric LPS as the active unit. The first is the observation that the diphosphoryl lipid A derived from the nontoxic LPS of *Rhodobacter sphaeroides* is biologically inactive, whereas the corresponding lipid A from the toxic LPS of *Escherichia coli* is active (Takayama et al., 1989; Kirkland et al., 1991; Qureshi et al., 1991a,b; Golenbock et al., 1991). The structural differences between these two compounds are the number of fatty acyl groups (five vs six) and the chain length of the hydroxy fatty acids (C_{10} vs C_{14}) (Qureshi et al., 1988a, 1991b). The former acts as an antagonist of the latter. For the fatty acyl chain length variations, it is difficult to rationalize how such a structural specificity can be achieved with aggregated LPS. Second, we have prepared a ReLPS–bovine serum albumin

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[1] Abbreviations: LPS, lipopolysaccharide; ReLPS, deep rough chemotype lipopolysaccharide; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; Kdo, 2-keto-3-deoxyoctonate.

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complex (designated peak II) which contains ReLPS in a highly disaggregated state (1–7 mol of ReLPS per mol of bovine serum albumin) (Takayama et al., 1990). Such a preparation was shown to activate 70Z/3 pre-B cells in the absence of serum.

If monomeric LPS is indeed the biologically active unit as suggested above, greater knowledge of its physical properties as the isolated monomer would be desirable. In this regard, only one study can be cited from the literature as being relevant. Previously, we examined some of the solubility properties of the ReLPS from *E. coli* (Takayama et al., 1990). We found that the solubility[2] of ReLPS in 150 mM Tris-HCl–KCl buffer, pH 7.5, at 37 °C was 2.8×10^{-8} M. An increase in neither the temperature (22 to 37 °C) nor the ionic concentration (0.75 to 150 mM) markedly changed the solubility of the ReLPS as would be expected for a micellar system. We have now examined the effect of pH on the solubility of ReLPS and have found that an increase in pH causes an increase in the solubility. The basis for this increase is the dissociation of tetraanionic ReLPS to pentaanionic ReLPS. We have also calculated the pK values of the phosphate groups of ReLPS. Due to the presence of the Kdo groups, these values are much higher than expected from compounds with only one phosphate group.

MATERIALS AND METHODS

Materials. HPLC grade methanol and chloroform were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). Silica gel H thin-layer plates (250 μm) were purchased from Analabs, Inc. (North Haven, CT). Tris (Trizma), MES, *trans*-cinnamic acid, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Triethylamine was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Sodium [1-^{14}C]acetate (40 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). All other chemicals used in this study were reagent grade.

Growth of Bacteria, Extraction of ReLPS, and Preparation of Purified ReLPS. Cells of *E. coli* D31m4 were grown in a New Brunswick 28-1 fermenter at 30 °C in LB broth medium (Difco Laboratories, Detroit, MI) as previously described (Takayama et al., 1983). ReLPS was extracted from these cells by the procedure of Galanos et al. (1969) with modifications as described by Qureshi et al. (1983), purified by the method described by Qureshi et al. (1988b), and dried under high vacuum to yield the ammonium salt of hexaacyl, bisphosphoryl ReLPS with a molecular weight of 2238, calculated as the free acid. Its structure is shown in Figure 1. Such a preparation was used to determine the nitrogen content and served as the reference standard for analytical thin-layer chromatography.

Nitrogen Analysis. We determined the total nitrogen content of the unlabeled ReLPS, prepared exactly like the radiolabeled ReLPS, by the semimicro Kjeldahl procedure

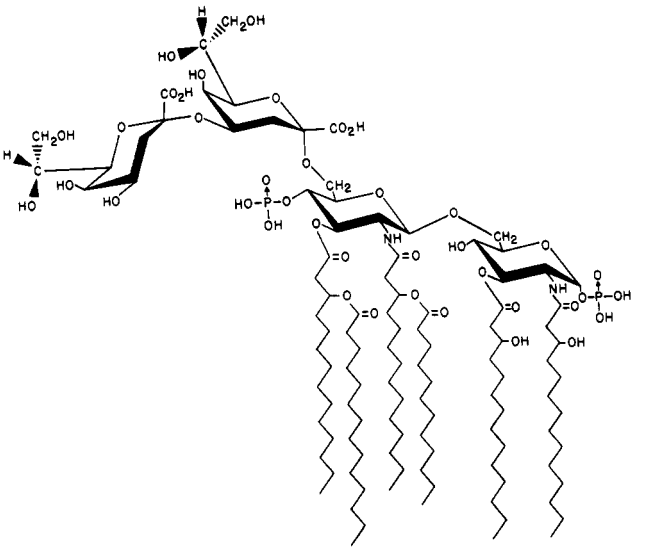


FIGURE 1: Chemical structure of the hexacyl, bisphosphoryl ReLPS from *E. coli* D31m4.

(Schulte et al., 1987). The purpose was to determine the precise molecular weight of the [^{14}C]ReLPS preparation used in this study. The DEAE-cellulose column purified and high vacuum dried ReLPS gave a nitrogen content of 2.20 ± 0.01%, which calculated out to 3.57 molar equiv of nitrogen. Thus when weighed, our [^{14}C]ReLPS preparations are expected to be the $(NH_4)_{1.6}$ salts with a molecular weight average of 2265.

Preparation of [^{14}C]ReLPS. A 160-mL culture of *E. coli* D31m4 was grown in LB broth with shaking at 30 °C to an absorbance of 0.100 at 600 nm. Then 2.0 mCi of sodium [1-^{14}C]acetate was added, and the culture was incubated to late log growth. The cells were then harvested by centrifugation and washed once with 0.9% saline. These cells were extracted as previously described (Takayama et al., 1990) to yield 13.2 mg of crude [^{14}C]ReLPS (5×10^7 cpm). This preparation was treated with 0.10 M EDTA, pH 7.0, and fractionated on a 2.2- × 24-cm DEAE-cellulose column (acetate form) using a linear gradient of 0.04–0.12 M ammonium acetate in chloroform/methanol/water (2:3:1, v/v) in a total volume of 500 mL (Takayama et al., 1990). Fractions (2.5 mL) were collected and analyzed for radioactivity. The [^{14}C]ReLPS peak appearing within fractions 97–112 was recovered by performing the chloroform/methanol/water two-phase extraction (Bligh & Dyer, 1959). The final yield of the ammonium salt of ReLPS was 4.0 mg with a specific radioactivity of 3.1×10^6 cpm/mg.

Suspension of [^{14}C]ReLPS in Buffer. Aqueous suspensions of [^{14}C]ReLPS in 50 mM Tris-HCl–100 mM KCl and 50 mM MES–100 mM KCl buffers were prepared by the methods of Takayama et al. (1990). [^{14}C]ReLPS (226 μg) was suspended in water containing 0.5% triethylamine to a concentration of 1.0 mg/mL and sonicated for 15 min. This sample was lyophilized and suspended in 1.00 mL of the desired buffer.

Equilibrium Dialysis Experiment. The solubility of the ReLPS suspension in buffer was determined on an equilibrium dialysis apparatus (Spectrum Medical Industries, Inc., Los Angeles, CA) at 22 °C and 7 rpm as previously described (Takayama et al., 1990). A Spectra/Por molecular-porous membrane filter (47-mm diameter) with a molecular cutoff of 6–8 kDa was used (Spectrum Medical Industries, Inc.). The sample, suspended in buffer, was loaded onto the donor side, whereas 0.80 mL of buffer was added to the acceptor side of the cell. After the cells reached equilibrium (24 h),

[2] In this paper, we define solubility as the saturation concentration of ReLPS in the monomeric state. The basic concept applied here is that amphiphiles at low concentration in aqueous medium do not aggregate appreciably and that, above the saturation concentration, any additional material is present as a dispersion of a solid. We prefer not to use the fashionable term, critical micelle concentration (CMC) (Mukerjee & Mysels, 1971; Lindman & Wennerström, 1980), because it suggests that monomeric amphiphile is in equilibrium with micellar amphiphile. In our experimental system, we believe that, above the solubility limit, monomeric ReLPS is in equilibrium with solid ReLPS. We have shown that the degree of solubilization of ReLPS is not affected by temperature or ionic concentration (Takayama et al., 1990) as would be expected for a micellar system.

the entire volume of the acceptor side of the cell was removed, lyophilized, and counted in a scintillation spectrometer. At least 5000 cpm were accumulated to achieve accuracy in counting of less than 1.4% SD. Fresh buffer was added to the acceptor side of the cell, and the procedure was repeated for 48- and 96-h equilibration periods. The solubility of [^{14}C]-ReLPS was measured in 50 mM Tris-HCl–100 mM KCl over a pH range of 6.50–8.20 and in 50 mM MES–100 mM KCl at pH 6.50. Here, MES was also used because pH 6.5 was just outside of the effective range of the Tris buffer. The method did not require a separation of excess suspended material from solution, a step that is extremely difficult owing to the highly sticky character of ReLPS (Takayama et al., 1990). Although the procedure caused a high level of adsorption to cell and membrane surfaces, it still allowed equilibrium to be established (Takayama et al., 1990). This procedure was capable of picking up 10^{-9} M concentrations of [^{14}C]ReLPS in 0.80-mL sample volumes when the [^{14}C]-ReLPS described above was used. The calculations of solubility were based on a molecular weight of 2265 for the ReLPS·$(NH_4)_{1.6}$.

Cinnamic acid was used as a model compound to test the equilibrium dialysis apparatus as a means of determining both solubility and pK value. To the donor side, containing 1.0 mL of 0.1 M sodium citrate (we generally varied the pH from 3.5 to 5.6), 20 mg of solid cinnamic acid was added, and the system, containing 0.8 mL of 0.1 M sodium citrate on the acceptor side, was equilibrated for 24 h at 22 °C. Then a 20-μL aliquot was removed from the acceptor side and transferred to 5.0 mL of 0.1 M HCl, and the absorbance was read at 280 nm on a Gilford UV spectrophotometer to determine the monomeric cinnamic acid concentration. The pH of the acceptor side was also determined. Under these conditions, the pH on the acceptor side rose by 0.04 unit within the lower range to a maximum level of 0.22 unit at the highest pH tested. The molar extinction coefficient for cinnamic acid in 0.1 M HCl was established to be 1.886×10^{-4} M. This experiment was repeated several times.

The fitting of the experimental data accumulated from this study to the equations was done by using linear and nonlinear regression analysis with the BMDP statistical programs on a VAX-11 computer (Digital Equipment Co., Maynard, MD) at the University of Wisconsin—Madison.

RESULTS AND DISCUSSION

Effect of pH on the Solubility of Cinnamic Acid. Cinnamic acid was used to demonstrate that both the solubility (monomeric concentration) and the pK could be measured for an appropriate known compound using the equilibrium dialysis apparatus. As shown in Figure 2A, the solubility of the cinnamic acid increased nonlinearly from 3.05×10^{-3} M at pH 3.57 to 2.14×10^{-2} M at pH 5.29. Equation 1 was found to fit the solubility data over this pH range as shown in Figure 2A.

$$S = S_0(1 + K_a/a_{H^+}\gamma^-) \quad (1)$$

Here K_a is the thermodynamic dissociation constant, and S_0 represents the solubility of the undissociated acid. This equation includes activity corrections appropriate for pH-related problems (Mukerjee & Moroi, 1978). The a_{H^+} value is 10^{-pH}, and γ^- is the activity coefficient of the anion which was estimated from the activity data of sodium propionate (Robinson & Stokes, 1959). The fitted value of S_0 was 2.54×10^{-3} M, and the pK was determined to be 4.54 with a 95% confidence limit of ±0.11. These values compare favorably with the published values for the solubility of cinnamic acid of about $3–6 \times 10^{-3}$ M and the pK of 4.44 at 25 °C (Dawson et al., 1986).

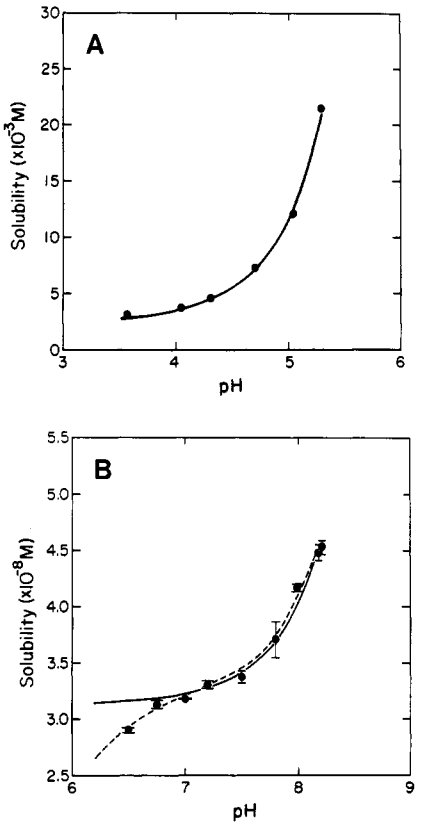


FIGURE 2: (A) Effect of pH on the solubility of cinnamic acid in 0.1 M sodium citrate buffer (pH 3.57–5.29). The observed values (solid circles; data from a representative experiment) are compared with the theoretically derived curve based on eq 1, $S = S_0(1 + K_a/a_{H^+}\gamma^-)$ (solid line). (B) Effect of pH on the solubility of ReLPS in 50 mM Tris-HCl–100 mM KCl buffer (pH 6.75–8.20) and 50 mM MES–100 mM KCl buffer (pH 6.50). The observed values (solid circles) are compared with the theoretically derived curves based on the simple eq 2, $S = S_0(1 + K/[H^+])$ (solid line), and the more complete eq 11, $C_T = S_0(1 + K_5/[H^+])/([H^+]/K_4' + 1)$ (broken line). The observed values are plotted with standard error of means bars.

Effect of pH on the Solubility of [^{14}C]ReLPS. Utilizing the equilibrium dialysis apparatus and procedures previously described (Takayama et al., 1990), we examined the effect of pH on the solubility of [^{14}C]ReLPS in two buffer systems. The 50 mM MES–100 mM KCl buffer was used for pH 6.30 and 6.50. The 50 mM Tris-HCl–100 mM KCl buffer was used for the pH range of 6.50–8.20. The solubility of [^{14}C]-ReLPS increased nonlinearly from $(3.20 \pm 0.01) \times 10^{-8}$ to $(4.55 \pm 0.07) \times 10^{-8}$ M when the pH was increased from 7.00 to 8.20 (Figure 2B). Although this increase in solubility was small, it was easily measurable by our procedure. The solubility of [^{14}C]ReLPS at pH 6.50 was lower than expected [$(2.92 \pm 0.02) \times 10^{-8}$ M] from a simple theoretical solubility curve. Similar results were obtained with both MES and Tris buffers at this pH. This observation will be discussed later.

The equilibrium dialysis method had a limited pH range (6.50–8.20) in the measurement of solubility of [^{14}C]ReLPS because it was labile at the higher and lower pH ranges. The radiolabeled ReLPS was stable for the 24- to 96-h equilibrium periods used only within this range. Measurements done at lower pH (pH 6.00 in Tris-HCl buffer and pH 6.30 in MES buffer) revealed that sufficient hydrolysis of the ^{14}C-labeled fatty acyl groups of ReLPS had occurred in 24 h, allowing the accumulation of fatty acids on the acceptor side of the

Table I: Distances between the Carboxylate Carbon Atoms of the Two Kdo Residues and the Phosphorus Atoms of the Two Phosphate Groups in ReLPS from *E. coli* as Determined by Molecular Modeling Techniques[a]

atoms	interatomic distance (Å)	
	range	in conformation c[b]
Kdo_1-C, Kdo_2-C	5.4–7.8	7.0
1-P, 4′-P	6–14	12.6
1-P, Kdo_1-C	8–13	11.7
1-P, Kdo_2-C	3–16	13.5
4′-P, Kdo_1-C	5–7.5	5.9
4′-P, Kdo_2-C	1–12	8.6

[a] The locations of the atoms involved in these distance calculations are shown in the partial space-filling molecular model of ReLPS (Figure 3). Abbreviations: 1-P, phosphorus of the reducing-end phosphate; 4′-P, phosphorus of the 4′-phosphate; Kdo_1-C, carbon of the carboxylate group of Kdo at the penultimate position; Kdo_2-C, carbon of the carboxylate group of Kdo at the nonreducing terminal. [b] These distances were computed for the most energetically favorable conformation of ReLPS (Kastowsky et al., 1991).

equilibrium dialysis cell and giving large solubility values. When measurements were made at higher pH (i.e., pH 8.50 in Tris-HCl buffer), based-catalyzed hydrolysis of the fatty acyl ester bonds occurred, to give larger solubility values for [^{14}C]ReLPS. In both cases, the presence of significant amounts of the free ^{14}C-labeled fatty acids on the acceptor side of the equilibrium dialysis cell was confirmed by analytical radio-thin-layer chromatography (data not presented). ReLPS appears to be stable only within the pH range 6.50–8.20.

Determination of Conformation and Intramolecular Distances of the Charged Groups in ReLPS by Molecular Modeling Techniques. The geometries of the ReLPS were analyzed at four different stages of calculation of the theoretical conformational analysis by molecular modeling as reported previously (Kastowsky et al., 1991). The conformations at the first stage, 809, the second stage, 264, the third stage, 41, and the fourth stage, 6, were available from this study. The conformations at the first and second stages resulted from simple combinations, so that most of these are energetically disfavorable. However, they reflect the maximum possible range of distances of the charged groups (Table I). Since the Kdo dimer is a relatively rigid structure, the distances between the carboxyl groups can only range from 5.4 to 7.8 Å. The 1-P to 4′-P and 1-P to Kdo_1-C distances showed intermediate variability, whereas the 1-P to Kdo_2-C and 4′-P to Kdo_2-C distances showed the widest range of variability. The 1-P to Kdo_1-C distance was limited to a minimum of 8 Å, indicating that 1-P cannot interact with Kdo_1-C, whereas all of the other phosphate to carboxyl interactions can occur. The 4′-P to Kdo_1-C distance was confined to a maximum of about 7.5 Å, so that in this region of the molecule the two charged groups formed a constant structural element of high charge density. Conformation c as described by Kastowsky et al. (1991) was chosen as the most energetically favorable conformation to use for this study. Using this conformation, we calculated the distances between the four charged groups in the molecule of ReLPS, and the values are given in Table I (also see Figure 3). As will be shown later, the influence of the Kdo carboxyl groups on the ionization of the two phosphate groups and the solubility of ReLPS is considerable.

Theoretical Basis for Solubility of ReLPS. When the pH of a suspension of [^{14}C]ReLPS was increased from 7.00 to 8.20 in Tris buffer, the solubility increased by only 42%. This suggested that at the lower pH values, a species A in solution is in equilibrium with the solid phase. As the pH is increased,

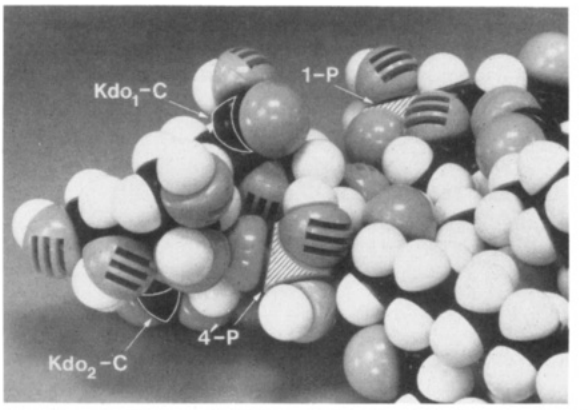


FIGURE 3: Partial spacing-filling molecular model showing the locations of the carbon atoms of the Kdo carboxylate groups (Kdo_1-C and Kdo_2-C) and the phosphorus atoms of the phosphate groups (1-P and 4′-P) in the ReLPS of *E. coli*. The rule used to construct the lipid A portion of the model is based on the assumption that hyperconjugation occurs between the carboxyl oxygen of the fatty acyl group and the proton of the sugar carbon to which the ester or amide group is linked (Chakrabarti & Dunitz, 1982; Schweizer & Dunitz, 1982).

a new species B is generated from species A by the dissociation of a single proton. The total solubility (S) of ReLPS is then the sum of the concentration of A and B. If K is the constant for the equilibrium $A \rightleftharpoons B + H^+$, then the solubility data, assuming constant activity coefficients, should be represented by eq 2,

$$S = S_0(1 + K/[H^+]) \quad (2)$$

where S_0 is the concentration of species A, which is constant, and $S_0K/[H^+]$ represents the pH-dependent concentration of species B. Equation 2 was found to fit the solubility data over the pH range 7.00–8.20 as shown in Figure 2B. The fitted value of S_0 was $(3.14 \pm 0.05) \times 10^{-8}$ M, and the value of K was $(2.86 \pm 0.16) \times 10^{-9}$ M, so that the pK was determined to be 8.54 with a 95% confidence limit of ±0.08. As discussed later, a more elaborate model for the entire pH range 6.50–8.20 leads to a slightly different pK value of 8.58.

Field Effects and the Ionization of the Phosphate Groups. Several important questions need to be addressed about the treatment outlined above. The first question is why only one dissociation step is involved when the ReLPS is expected to be a hexabasic acid. The second question concerns the observed high pK value of 8.54 obtained from the data in the pH range 7.00–8.20. For comparison, we note that the pK_1 and pK_2 values of individual phosphate groups in glucose and glucosamine phosphates are about 1.0 and 6.1, respectively (Dawson et al., 1986), and the pK values of individual carboxylate groups in model compounds are less than 3 (Stecher, 1968; Gordon & Ford, 1972). A third question is why the solubilities at pH values lower than 7 are somewhat less than those predicted by eq 2. We believe that these questions are related and that the answers are to be found in the field effects of charged groups in raising the pK values of other dissociable groups (Edsall & Wyman, 1958; Gould, 1959) and in additional factors related to the nature of the "solid" phase of ReLPS.

Preexisting negative charges increase the local concentrations of protons around other nearby and dissociable groups through an electrostatic effect, thus reducing the dissociation constant measured. Consider the simplest example of symmetrical dibasic acids (Edsall & Wyman, 1958; Kirkwood & Westheimer, 1938a,b; Westheimer & Shookhoff, 1938), where the differences between pK_2 and pK_1 of such acids can be

$$P_1^-\text{-}P_4^-\text{-}C_1^-\text{-}C_2^- \underset{}{\overset{k_a}{\rightleftharpoons}} P_1^=\text{-}P_4^-\text{-}C_1^-\text{-}C_2^- + H^+$$

$$P_1^-\text{-}P_4^-\text{-}C_1^-\text{-}C_2^- \underset{}{\overset{k_b}{\rightleftharpoons}} P_1^-\text{-}P_4^=\text{-}C_1^-\text{-}C_2^- + H^+$$

$$P_1^=\text{-}P_4^-\text{-}C_1^-\text{-}C_2^- \underset{}{\overset{k_c}{\rightleftharpoons}} P_1^=\text{-}P_4^=\text{-}C_1^-\text{-}C_2^- + H^+$$

$$P_1^-\text{-}P_4^=\text{-}C_1^-\text{-}C_2^- \underset{}{\overset{k_d}{\rightleftharpoons}} P_1^=\text{-}P_4^=\text{-}C_1^-\text{-}C_2^- + H^+$$

FIGURE 4: Microscopic dissociation of tetraanionic ReLPS. Notations used for the ionizable groups of ReLPS: P_1 and P_4, phosphate groups at the 1-position and the 4′-position, respectively; C_1 and C_2, carboxylate groups of the penultimate and terminal Kdo groups of the dissacharide, respectively. We propose that, at neutral pH, the ReLPS is primarily in the tetraanionic form, which dissociates according to the four equilibria shown with increase in pH.

explained reasonably well by the theory of Kirkwood and Westheimer (1938a,b), which leads to the equation

$$\Delta pK = pK_2 - pK_1 = Ne^2/2.303RTDr + \log 4 \quad (3)$$

Here log 4 represents a statistical factor appropriate for symmetrical dibasic acids such as succinic acid, N is Avogadro's number, e is the protonic charge, R is the molar gas constant, T is the absolute temperature, r is the distance between the dissociable groups, and D is the "effective" dielectric constant. At 25 °C, the electrostatic factor in eq 3 can be represented as $243/Dr$, where r is in Å. The value of D differs from the solvent value (78.5) because the electrostatic effects are transmitted partly through the organic molecules which provide a medium of low dielectric constant. Westheimer and Shookhoff (1938) showed that the experimental ΔpK of malonic acid (2.86) requires a D value of 26 and an r value of 4.1 Å. For succinic acid, the ΔpK of 1.44 corresponds to a D value of 50 and an r value of 5.75 Å.

The low pK_1 values of the phosphate groups (about 1.0) and the carboxylate groups (2.5 in the model compound, 2-oxobutanoic acid) (Dawson et al., 1986) make it highly likely that ReLPS in solution has at least 4 negative charges at a solution pH of about 7. Thus in our experiments we are likely to be dealing with the second dissociation of one or both of the phosphate groups, i.e., pK_5 or pK_6, for ReLPS. To examine whether the pK value is compatible with pK_5 or pK_6, we have calculated some field effects for the fifth and sixth dissociations of ReLPS. The dissociation equilibria of species carrying four charges, designated $P_1^-\text{-}P_4^-\text{-}C_1^-\text{-}C_2^-$, can be considered as shown in Figure 4. The relationship between the k_a, k_b, k_c, and k_d values, representing the microscopic dissociation constants of the individual groups to the overall K_5 and K_6, which are the fifth and sixth dissociation constants of ReLPS, are shown below:

$$K_5 = k_a + k_b = \frac{[P_1^{2-}\text{-}P_4^-\text{-}C_1^-\text{-}C_2^-][H^+] + [P_1^-\text{-}P_4^{2-}\text{-}C_1^-\text{-}C_2^-][H^+]}{[P_1^-\text{-}P_4^-\text{-}C_1^-\text{-}C_2^-]} \quad (4)$$

$$1/K_6 = 1/k_c + 1/k_d = \frac{[P_1^{2-}\text{-}P_4^-\text{-}C_1^-\text{-}C_2^-] + [P_1^-\text{-}P_4^{2-}\text{-}C_1^-\text{-}C_2^-]}{[P_1^{2-}\text{-}P_4^{2-}\text{-}C_1^-\text{-}C_2^-][H^+]} \quad (5)$$

We now assume that these microscopic dissociation constants are a combination of the intrinsic pK_2 values of the individual phosphate groups, assumed to be 6.1, and the field effects resulting from the presence of other charged groups. The distances between the charged groups in ReLPS are estimated from conformation c (Table I). We have used the distances between phosphorus and carbon atoms. These are, in Å, 12.6 for 1-P to 4′-P, 11.7 for 1-P to Kdo_1-C, 13.5 for 1-P to Kdo_2-C, 5.9 for 4′-P to Kdo_1-C, and 8.6 for 4′-P to Kdo_2-C. The Kdo_1-C to Kdo_2-C distance was not needed in our calculations.

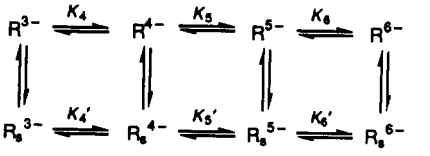
The charge effect of any charged group on the pK value of any other dissociable group can be calculated to a sufficient degree of approximation from the formula $243Z/Dr$, where Z is the number of charges on the charged group, r is the distance in Å, and D is the "effective" dielectric constant. As an example, if D is 25, the microscopic pk_a value for the P_1^- to P_1^{2-} dissociation of $P_1^-\text{-}P_4^-\text{-}C_1^-\text{-}C_2^-$ can be calculated from the distance parameters of conformation c of ReLPS to be

$$pk_a = 6.1 + (243/25)(1/12.6 + 1/11.7 + 1/13.5) = 8.42 \quad (6)$$

These calculations can only be of semiquantitative significance because of the uncertainty of the estimated distances between the charges and the value of the effective dielectric constant, D. The value of D may well be different from interactions between different charged groups. If a mean value of 23.2 is chosen for D along with the distance parameters of the charged groups in conformation c of ReLPS as shown in Table I to calculate k_a, k_b, k_c, and k_d, the pK_5 is calculated to be 8.58 using k_a and k_b (eq 4), and pK_6 is calculated to be 10.76 using k_c and k_d (eq 5). For this chosen value of D, the experimental pK value agrees with pK_5, and pK_6 is high enough to be out of the experimental pH range as observed. If a mean D value of 44.1 is chosen, pK_5 and pK_6 are calculated to be 7.34 and 8.58, respectively. Here pK_6 agrees with the experimental value, but pK_5 is within the experimental pH range 7.0–8.2, suggesting that there are two dissociation processes occurring within this pH range. This is not observed. Thus, although the field effects are compatible with the identification of the experimental pK as either pK_5 or pK_6, it seems that pK_5 may be a better assignment for the observed ionization. Further arguments to support this assignment are presented later.

Nature of the Solid Phase. The dimensions of the ReLPS particles obtained from electron microscopy show that these particles are relatively small with a large surface area (Takayama et al., 1990). The average particle has dimensions of roughly 25 × 25 × 70 nm³. The number of molecules in such a particle is high (about 12 000), but some 40% of the particles are likely to be in the surface layer. These molecules must have polar portions that are exposed to the aqueous layer and partly ionized. We therefore need to consider the solubility equilibrium in terms of equilibrium of the solution species with the surface species.

We assume that around pH 7 the predominant species both in solution and at the solid surface are ReLPS molecules carrying four charges. The equilibria between the various charged species in solution and at the solid surface at pH 6.5 and higher can be represented as



Here R represents ReLPS in solution, and R_s represents ReLPS at the surface of the solid particles. K_4, K_5, and K_6 represent

the overall dissociation constants in solution. K_4', K_5', and K_6' represent dissociation constants of ReLPS molecules at the surface of the solid particles in equilibria such as

$$R_s^{4-} \overset{K_5'}{\rightleftharpoons} R_s^{5-} + H^+$$

If the solid surface is exclusively in the R_s^{4-} form, the equilibrium concentration of R^{4-} in solution is defined as S_0. When the solid surface contains other forms of R such as R_s^{3-}, the fraction of R_s^{4-} molecules at the surface, represented by f^{4-}, will be less than unity. The R^{4-} concentration in solution will then be given by $[R^{4-}] = S_0 f^{4-}$. The value of f^{4-} will depend on the pH and the values of K_4', K_5', and K_6'. For example, the above equilibrium for R_s^{4-} leads to the relationship $K_5' = f^{5-}[H^+]/f^{4-}$. Since $f^{3-} + f^{4-} + f^{5-} + f^{6-} = 1$, the value of f^{4-} is given by the equation

$$f^{4-} = 1/([H^+]/K_4' + 1 + K_5'/[H^+] + K_5'K_6'/[H^+]^2) \quad (7)$$

On the other hand, the total ReLPS concentration in solution, C_T, can be represented as $C_T = [R^{3-}] + [R^{4-}] + [R^{5-}] + [R^{6-}] = [R^{4-}]([H^+]/K_4 + 1 + K_5/[H^+] + K_5K_6/[H^+]^2)$. Since $[R^{4-}] = S_0 f^{4-}$, the full expression for the total solubility, C_T, is given by

$$C_T = S_0([H^+]/K_4 + 1 + K_5/[H^+] + K_5K_6/[H^+]^2)/$$
$$([H^+]/K_4' + 1 + K_5'/[H^+] + K_5'K_6'/[H^+]^2) \quad (8)$$

It should be noted that the solubility of any charged form other than R^{4-} can be represented by S_0 for R^{4-} and the appropriate K values in solution and at the surface. For example, the solubility of R^{5-}, S', can be represented as $[R^{5-}]/f^{5-}$. Since $[R^{4-}]/f^{4-}$ represents S_0, $S' = S_0K_5/K_5'$. Similarly, the solubility of R^{3-}, S'', can be represented as S_0K_4'/K_4. As will be discussed later, K_5' and K_4' have lower values than K_5 and K_4, so that the solubility of R^{5-}, S', is greater than the solubility of R^{4-}, S_0, whereas the solubility of R^{3-}, S'', is less than S_0.

The calculated concentration of solid particles of ReLPS on the donor side of the equilibrium dialysis apparatus is small (<17 nM, or a volume fraction of $<2 \times 10^{-4}$). Thus the Donnan effect (Overbeek, 1956) is negligible and not a problem in the interpretation of our results.

Application of Full-Expression Equation 8. Equation 8 indicates that if the solution dissociation constants are identical with those operative for the solid surface, then C_T should equal S_0 at all pH values. However, K_s values are expected to be much lower than K values for two reasons: (a) The dissociable groups in the solid phase are in an interfacial environment where the effective polarity of the microenvironment is lower than that for the solute species in solution (Mukerjee & Ray, 1966; Mukerjee et al., 1977; Ramachandran et al., 1982). (b) The presence of numerous negative charges at the interface produces a negative electrostatic potential, ψ, which increases the local concentration of H^+. These two factors can be combined in the following equation as suggested some years ago (Mukerjee & Banerjee, 1964)

$$pK' = pK'_{(i)} + \psi/59 \quad (9)$$

Here, the apparent pK' of the interfacial species is determined by the intrinsic $pK'_{(i)}$ at the interface, which is the value of pK' when $\psi = 0$, and the $\psi/59$ factor, where ψ is the absolute value of the surface potential in mV and 59 is the appropriate Nernst-type factor for 25 °C. Thus pK' increases by one unit when the value of ψ increases by 59 mV.

For quantitative purposes, we note that for some anionic indicator dyes in nonionic micelles, for which $\psi = 0$, the medium effects at the interface can make $pK'_{(i)}$ higher than pK in solution by 1–2 units (Mukerjee et al., 1977). With respect to an estimate of ψ, the Gouy–Chapman theory for planar electrical double layers (Mukerjee & Banerjee, 1964; Vold & Vold, 1983) can be used. At 25 °C in aqueous media, ψ in mV can be determined from the equation

$$\psi = 51.4 \sin[h^{-1}(135/Ac^{1/2})] \quad (10)$$

where A is the area in Å^2 per unit charge at the surface and c is the molar concentration of the univalent electrolyte in solution. When ReLPS is in the tetraanionic state, the average area per unit charge of closely packed molecules in the solid phase is about 37 Å^2, whereas c is 0.15 M in this experiment. The calculated ψ is about 151 mV, so that $\psi/59$ is 2.56. It was shown some years ago (Mukerjee & Benerjee, 1964) that ψ calculated by this theory gives an overestimate. Nevertheless, the combined effect of the two factors should make pK' values some 2 or more units higher than the pK values.

Equation 8 becomes much simpler in the pH range 6.5–8.2 in view of the above argments. We have proposed that our pK value of 8.58 corresponds to pK_5. pK_6 then is much higher, and the term containing K_6 in eq 8 can then be ignored. For reasons discussed before, pK_4 is likely to be less than 4–5, so that the term $[H^+]/K_4$ in eq 8 is negligible in the pH range 6.5–8.2. Since pK_5' and pK_6' are likely to be significantly higher than pK_5 or pK_6, those terms containing K_5' and K_6' can also be ignored. Equation 8 thus simplifies to

$$C_T = S_0(1 + K_5/[H^+])/([H^+]/K_4' + 1) \quad (11)$$

When this equation was used to fit the experimental data over the whole range, an excellent fit was obtained (Figure 2B) with $S_0 = (3.23 \pm 0.06) \times 10^{-8}$ M. The estimated value of pK_5 was 8.58 ± 0.08 (95% confidence level), a value that is only slightly different from the previous estimate of 8.54 based on the simple eq 2. Of particular interest is the value for pK_4' of 5.5 ± 0.3 which should represent the dissociation of a Kdo carboxyl group. The intrinsic pK value for a carboxyl group associated with a Kdo group is expected to be about 2.5 from the pK value of 2-oxobutanoic acid. The combination of the charge effects and the interfacial microenvironmental effects has raised this value by about 3 units, which is not unreasonable. Thus the reason for the reduction of the apparent solubility at pH values less than 7 is the formation of the less soluble R_s^{3-} species at low pH values. It is to be noted that if our pK value of 8.58 is considered to be pK_6, then this pK_4' must be treated as pK_5'. A value of 5.5 here is much too low for the second dissociation of a phosphate group considering that pK_2 of the model glucose and glucosamine phosphate is 6.1 in the absence of any charge effects. These arguments, therefore, provide additional justification for assigning the experimental pK value of 8.58 to pK_5.

An interesting consequence of eq 9 is that the rate of increase in the solubility of ReLPS with increase in pH levels off at high pH values. At very high pH values, the solubility is expected to increase by a factor of $K_5K_6/K_5'K_6'$ over S_0, independent of pH. These high pH values are of no physiological significance. Moreover, ReLPS has poor chemical stability at high pH and may undergo self-association in solution when the solubility is high, so that it may be difficult to study. Nevertheless, some orders of magnitude are of interest. If pK_5' and pK_6' are each 2 units lower than pK_5 and pK_6, the monomer solubility at high pH would be $10^4 S_0$, i.e., 3.2×10^{-4} M, or about 0.7 mg/mL. On the other hand, if the primary species at pH 7.0 in solution is $ReLPS^{5-}$, the maximum solubility would increase by K_6/K_6', to about 0.007

mg/mL, which seems to be too low. These calculations lend additional support to the identification of our pK value as pK_5.

Ionic States of Lipid A. The influence of the charged Kdo groups on ReLPS is significant. If no Kdo group is present, as is the case of diphosphoryl lipid A, pK_3 (the third dissociation constant of the phosphate groups) would be lower than in ReLPS but higher than 6.1 due to the field effect of one phosphate group on the other. These two values can now be calculated for lipid A by the method outlined for ReLPS. Assuming that $D = 23.2$, the microscopic pK values would be pK_a = pk_b = 6.93 and pk_c = pk_d = 7.76. The dissociation equilibria for lipid A are similar to those for ReLPS except for the absence of the two carboxylate groups, C_1 and C_2 (Figure 4). Since $k_a + k_b$ would equal K_3 in eq 4, pK_3 would be 6.63. Since $1/K_4 = 1/k_c + 1/k_d$ from eq 5, pK_4 would be 8.06. Thus, at the near physiological pH of 7.4, the dianionic form would be 12%, the trianionic form, 72%, and the tetraanionic form, 16%, of the total lipid A content. The predominant trianionic form would be represented by an equal mixture of P_1^{2-}-P_4^- and P_1^--P_4^{2-} with one of the phosphate groups carrying two charges. At this pH, the distribution of the ionic states of ReLPS would be quite different: tetraanionic form, 94%; pentaanionic form, 6%; hexaanionic form, <0.01%. The predominant tetraanionic form, P_1^--P_4^--C_1^--C_2^-, has only single charges on the phosphate groups. In the pentaanionic form, the ratio of P_1^{2-}-P_4^--C_1^--C_2^- to P_1^--P_4^{2-}-C_1^--C_2^- would be 21:1. Although these calculations are sensitive to the particular values chosen for several of the parameters, we feel that the pK values obtained are good estimates.

Concluding Remarks. We have essentially confirmed the solubility results obtained previously for the ReLPS (Takayama et al., 1990). We have now shown that in the pH range tested (6.50–8.20) the tetraanionic ReLPS is in equilibrium with the pentaanionic form. This mean that the two carboxylate groups of the two Kdo moieties in the tetraanionic form are completely dissociated and that each of the two phosphate groups is monoanionic. With an increase in pH, there is an increase in the pentaanion fraction. The ReLPS at pH 7.4 (near physiological) and 22 °C has a solubility value of $(3.39 \pm 0.05) \times 10^{-8}$ M.[3] Only 6% of ReLPS is in the pentaanionic form at this pH. The concentration of monomeric ReLPS is high enough to fully activate 70Z/3 pre-B cells, which are the least sensitive of the responding cells (Takayama et al., 1990). It gives us a reference point in estimating the solubility of other LPSs (the rough chemotype series of LPS and the smooth LPS) as well as lipid A. As the size of the polysaccharide moiety increases, one would expect LPS solubility to increase, whereas lipid A is likely to be less soluble than ReLPS. It is only the magnitude of these differences than remains uncertain. It would now be desirable to determine the solubility of both mono- and diphosphoryl lipid A. This study thus establishes the basic solubility properties of a highly active and well-characterized model LPS. Such information should be useful in the study of the first step, release of LPS into solution, and the second step, binding to receptors (Lei & Morrison, 1988; Hampton et al., 1988; Hara-Kuge et al., 1990; Kirkland et al., 1990: Dziarski, 1991), to the LPS binding protein (LBP) (Tobias et al., 1986; Raetz et al., 1991), or to septin (Wright et al., 1992), in the activation of immune cells of the mammalian system.

Since the buffer system used in these dialysis experiments contained only monovalent cations (and not divalent cations), it might be argued that, in the biological systems, the solubility and perhaps other properties of ReLPS might be different from the experimental situation. Indeed, it is well established that different salt forms of LPS and lipid A can show considerable differences in solubility, from the Ca^{2+} and Mg^{2+} salts (turbid solutions), to the Na^+, K^+, NH_4^+, pyridinium, and ethanolammonium salts (opalescent solutions), to the triethylammonium salt (a clear solution) (Galanos & Lüderitz, 1975). Such a clear solution of ReLPS is still highly aggregated (Takayama et al., 1990). However, even under such conditions, there must be a finite concentration of monomeric LPS. This is suggested from our previous study to determine the solubility of ReLPS in the culture medium for the activation of the 70Z/3 pre-B cell line, which contained an abundance of divalent cations (Takayama et al., 1990). At least for such a species, it appears justified to extrapolate our experimental data to the complex biological systems. Nevertheless, the possible effects of divalent cations on the monomeric concentration and the biological activities of LPS remain to be studied.

When we compared the pK values of ReLPS to diphosphoryl lipid A, it became clear that the ionic properties of these two glycolipids are quite different. At the near physiological pH of 7.4, we calculated that 94% of the ReLPS is in the tetraanionic form (containing two monoanionic phosphates). In contrast, only 12% of the diphosphoryl lipid A was calculated to be in the dianionic form (similarly with two monoanionic phosphates). This represents an 8-fold higher proportion of the monoanionic phosphate-containing glycolipid in ReLPS as compared to the diphosphoryl lipid A. A great amount of charge on the phosphate groups, which are close to the hydrophobic chains that are probably responsible for binding to the membrane and receptors, is likely to reduce the binding and, perhaps, cause changes in the conformation of the bound ReLPS. If one considers that lipid A is the biologically active moiety of LPS and that Kdo is inactive, this difference in the monoanionic phosphate-containing forms might be the reason for the observed 10-fold greater biological activity of ReLPS over the diphosphoryl lipid A (Takayama & Qureshi, 1992). Loppnow et al. (1989) showed that the LPSs have 10- to 100-fold greater interleukin-1-inducing capacity than the diphosphoryl lipid A's in human mononuclear cells. Kovach et al. (1990) showed that the ReLPS is 11-fold more active than diphosphoryl lipid A in the induction of tumor necrosis factor release by human whole blood. This may point to a new concept in the structure-to-function relationship that Kdo groups (and possibly other nearby charged groups) in the LPS molecule can modulate the biological activity of the lipid A moiety by controlling its ionic state.

[3] The slightly higher solubility value given here as compared to the previous study (Takayama et al., 1990) is due to a correction made in the M_r value of the [^{14}C]ReLPS used. Previously, we incorrectly assumed that the preparation was a hexaammonium salt instead of a mono- to diammonium salt.

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