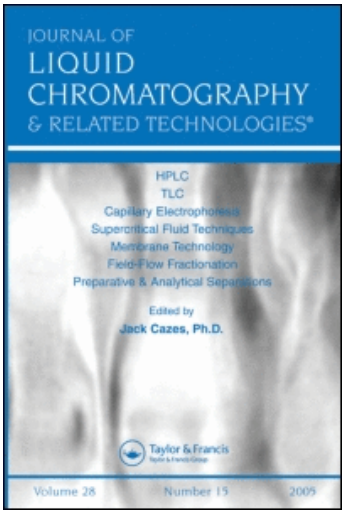

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Countercurrent Chromatographic Separation of Bacteria with Known Differences in Surface Lipopolysaccharide

Loretta Leive[a]; Loretta M. Cullinane[a]; Yoichiro Ito[b]; Gregory T. Bramblett[b]
[a] Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Bethesda, Maryland [b] Laboratory of Technical Development, National Heart, Lung, and Blood Institute, Bethesda, Maryland

To cite this Article Leive, Loretta , Cullinane, Loretta M. , Ito, Yoichiro and Bramblett, Gregory T.(1984) 'Countercurrent Chromatographic Separation of Bacteria with Known Differences in Surface Lipopolysaccharide', Journal of Liquid Chromatography & Related Technologies, 7: 2, 403 — 418
To link to this Article: DOI: 10.1080/01483918408073975
URL: http://dx.doi.org/10.1080/01483918408073975

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 403-418 (1984)

COUNTERCURRENT CHROMATOGRAPHIC SEPARATION OF BACTERIA WITH KNOWN DIFFERENCES IN SURFACE LIPOPOLYSACCHARIDE

Loretta Leive and Loretta M. Cullinane
Laboratory of Biochemical Pharmacology
National Institute of Arthritis, Diabetes, and
Digestive and Kidney Diseases
Building 4, Room 116
and
Yoichiro Ito and Gregory T. Bramblett
Laboratory of Technical Development
National Heart, Lung, and Blood Institute
Building 10, Room 5D12

National Institutes of Health
Bethesda, Maryland 20205

ABSTRACT

Countercurrent chromatography was used to separate cells of Salmonella typhimurium whose surfaces were identical except for the proportion of lipopolysaccharide molecules with long versus short chain polysaccharide chains. Large differences in partition properties resulted from slight differences in polysaccharide composition, and the results suggest that the physical state of the molecules, as well as their composition, can affect the partition of such cells.

INTRODUCTION

As is well known, countercurrent distribution with suitable polymer phase systems can separate particles on the basis of charge and hydrophobicity (1, 2). The recently described methods of countercurrent chromatography (3, 4) should provide a powerful method of separating particles that differ only slightly in the hydrophobicity and/or charge of their surfaces, and testing the effect of slight variation in the macromolecules of the cell

 0148-3919/84/0702-0403$3.50/0

surface on these properties. To test this possibility, we used Salmonella typhimurium, which normally produces a surface completely covered with lipopolysaccharide (LPS) molecules containing long carbohydrate chains [see (5) for review of LPS structure]. Mutants defective in UDP galactose-4-epimerase cannot synthesize galactose, a sugar required for synthesis of the complete polysaccharide of S. typhimurium, and therefore in the absence of added galactose will make LPS molecules with a very short chain polysaccharide. If galactose is then added, LPS molecules synthesized subsequently will have a long polysaccharide (6). Stendahl and coworkers (7-9), using countercurrent distribution, were the first to note that LPS determines the partition of S. typhimurium on countercurrent distribution, so that bacteria covered with long chain LPS partition as hydrophilic uncharged particles, and those covered with short chain LPS partition as hydrophobic, charged particles. They also found that growth of these mutant bacteria in a medium containing galactose resulted in a change in the elution properties of the cells from a hydrophobic charged particle to one which was hydrophilic and neutral (10). Since synthesis of LPS is continuous during steady-state growth of the bacteria, we reasoned that growth of mutant bacteria in the presence of galactose for various times should provide particles with defined percentages of long chain LPS, and thus defined proportions of both types of LPS on their surfaces. We would thus be able to test the resolving power of the countercurrent chromatography apparatus with particles of known, slightly different properties and also determine whether the distribution of the particles reflects solely their LPS composition.

We show herein that when we fractionate bacteria that have increasing percentages of long chain LPS, there are discontinuous changes in the position of these bacteria that do not reflect solely their percentages of surface constituents. Some bacteria containing as little as 20% long chain LPS fractionate at the position of bacteria containing 100% long chain LPS. We postulate

that the distribution of the LPS on the surface of the cell and not solely its composition can also affect partition in countercurrent chromatography.

MATERIALS AND METHODS

Reagents

[3,4,5-^{3}H]Leucine (110 Ci/mmol) was from New England Nuclear Co. (Boston, Massachusetts). [1-^{14}C]Galactose was from New England Nuclear Co. (56.5 mCi/mmol) or Amersham-Searle Co. (Arlington Heights, Illinois) (49.4 mCi/mmol). Proteose Peptone No. 3 and beef extract were from Difco Laboratories (Detroit, Michigan). Polyethylene glycol 6000 was obtained from Union Carbide Corp. (Oak Ridge, Tennessee) and dextran 500 from Sigma Chemical Co. (St. Louis, Missouri). All other reagents were from standard sources.

Growth of Bacteria

S. typhimurium G30, a mutant lacking UDP galactose-4-epimerase, was the kind gift of Dr. Paul Rick, Uniformed Services University of the Health Sciences, Bethesda, Maryland. It was grown at 37°C with shaking on proteose peptone beef extract medium (PPBE) (11) which had been depleted of residual galactose by allowing the bacteria to grow for several generations, centrifuging down the bacteria, and sterilizing the medium by filtration. Fresh bacteria that had been grown only on depleted PPBE were then used for the experiment. Galactose was added as indicated for each experiment. When galactose was to be added for only short times, the cells were previously grown for several generations in the presence of 0.01 mM D-fucose, which induces the synthesis of enzymes of galactose transport and metabolism. Radioactive galactose to label LPS and radioactive leucine to label protein were added as indicated for each experiment. Cells were grown to between 2 and 6 x 10^{8} bacteria per ml, as measured by OD_{530} after calibration of optical density to bacterial counts by standard methods. Cells were harvested by centrifugation at 8,000 x g for 5-10 minutes at 4°C in the presence of 0.005% NaN_3

to prevent further growth and metabolism. Samples were washed with 0.03 M Tris-chloride, pH 7.0, containing 0.005% NaN_3, and resuspended in the same buffer at 4 or 5 times the initial cell concentration. Fractions were often precipitated in trichloroacetic acid (5%) prior to counting in a scintillation counter; for all samples other than those labeled for < 3 minutes in galactose, total and acid-precipitable counts were virtually identical.

Apparatus

Countercurrent chromatography was performed with the most recent model of the nonsynchronous flowthrough coil planet centrifuge without rotating seals (4). The apparatus subjects the coiled column assembly to a planetary motion which consists of slow rotation (0-50 rpm) around its own axis and high speed revolution (500-1,000 rpm) around the central axis of the apparatus. The revolution produces a strong centrifugal force field (maximum 150 x g) while the slow rotation of the coil assembly provides efficient mixing of the two polymer phases and retention of the stationary phase in the coil. Both revolutional and rotational speeds of the coil are made independently adjustable to meet the requirements for the aimed separation. The coiled column was prepared from a single piece of 1 mm i.d. PTFE tubing (Zeus Industrial Products, Raritan, New Jersey) by winding it onto 6 units of 20 cm long, 0.6 cm o.d. stainless steel pipe cores in a series to make about 600 helical turns with a total capacity of approximately 15 ml. Both inlet (head) and outlet (tail) terminals of the coil were each connected to a piece of 0.4 mm i.d. PTFE tubing which was led to the outside of the rotor without the use of rotating seals. This seal-free rotor design makes the entire elution system leak-proof to provide a safeguard in separation of hazardous agents.

Preparation of Polymer Phase System

The polymer phase system employed in this study is based on the work of Stendahl et al. (7-9) and is composed of 6.2% (w/w)

dextran 500, 4.4% (w/w) polyethylene glycol 6000, 0.05 M Tris-chloride (pH 7.0), 10 mM potassium phosphate (pH 7.0), and 0.01% sodium azide. The solvent system was prepared by mixing equal volumes of the following two stock solutions (A and B) in a separatory funnel at room temperature. Stock solution A (double strength polymer solution) was prepared by dissolving 124 g of dextran 500 and 88 g of polyethylene glycol 6000 in 788 g of warm distilled water. Stock solution B (double strength buffer solution) was prepared by mixing 200 ml of 0.5 M Tris-chloride (pH 7.0), 200 ml of 0.1 M potassium phosphate (pH 7.0), and 20 ml of 1% sodium azide, in a 1-liter volumetric flask and adding water to bring the final volume to 1 liter. Stock solution A, because it forms two layers, should be thoroughly mixed into homogeneous suspension before each use. Strictly speaking, this method gives a concentration of each polymer slightly lower than the nominal value (on the order of 0.1%). However, it is simple and gives highly reproducible results.

Separation Procedure

Separation of bacterial cells was performed as follows: The coiled column is first filled with approximately equal amounts of the upper and the lower phases of the polymer phase system described above. This is done by delivering the solvents from a horizontally positioned syringe (20 ml capacity) containing 10 ml of each phase. After the filling process is completed, 1 ml of the sample suspension in equal amounts of the two phases is injected into the column through the sample port located at the pump outlet. Then the apparatus is run at 600 rpm combined with a slow column rotation of 5 rpm while the upper mobile phase is pumped into the column at a rate of 8.5 ml/h with an FMI Lab Pump (Fluid Metering Inc., Oyster Bay, New York). The effluent from the outlet of the column is collected with an LKB fraction collector to obtain 1 ml fractions for further analysis. After the desired number of fractions (30 fractions) are collected, the centrifuge run is terminated and the column contents (15 ml) are further

fractionated by eluting the stationary column with an equal volume mixture of the two phases with a syringe driver as described in filling the column. Then the column is emptied by pushing with air followed by washing with distilled water. The column is finally filled with 1 N NaOH and left overnight. This column cleaning procedure prevents adhesion of cells to the column wall, resulting in near 100% cell recovery.

RESULTS

Stendahl and coworkers devised a method for separating S. typhimurium that contain long chain LPS from those that contain short chain LPS on countercurrent distribution (7); in their work, the peaks containing the two types of cells were separated by 15 fractions. When we used their solvents to separate such cells by countercurrent chromatography, the cells containing long chain LPS distributed almost entirely into the mobile phase as expected and eluted out quickly from the column, but cells with short chain LPS remained in the column, even after chromatography was carried out for several days or 500 fractions were collected (data not shown). For this reason, it was necessary to modify the method: After the cells containing long chain LPS had been eluted and 30 fractions collected, the centrifuge run was terminated and the column contents containing retained cells were fractionated as described earlier. The results are shown in Fig. 1. Very sharp peaks, corresponding to cells containing long chain LPS (designated peak I, at left) and cells containing short chain LPS (designated peak II, at right) were obtained. There was no overlap, and 93-98% of the cells put into the column were recovered, as measured by recovery of radioactivity.

Having obtained a clear separation, we tested the effect of slight differences in the proportion of long chain LPS on the elution properties of the bacteria. During steady-state logarithmic growth at a given temperature, S. typhimurium synthesizes LPS continuously, and the ratio of LPS molecules to cell protein is known (12). When S. typhimurium G30 is transferred

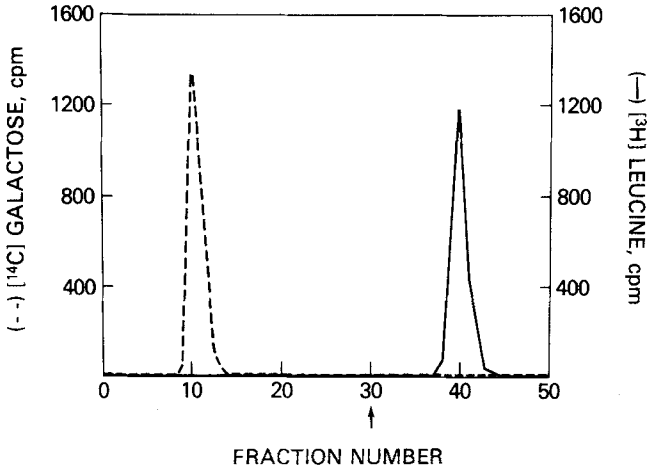

FIGURE 1. Separation of cells of S. typhimurium G30 containing long chain LPS from those containing short chain LPS by countercurrent chromatography. Cells were grown either without galactose and with [^{3}H]leucine (1 μCi/ml), or in the presence of 0.1 mM [^{14}C]galactose (1 mCi/mmol). The cells were harvested and washed, and 3 x 10^{9} cells of each were mixed and subjected to countercurrent chromatography as described. In this and other figures, the arrow on the bottom axis indicates the point at which the centrifuge run was terminated to start fractionation of the column contents. Also, the left peak will be referred to as peak I and the right peak as peak II. Identical results were obtained if the culture containing galactose was labeled with [^{14}C]leucine instead of [^{14}C]galactose.

from media lacking galactose, where it makes short chain LPS, to one containing galactose, all newly synthesized molecules of LPS have long chain polysaccharide, but pre-existing short chain LPS molecules are not modified or converted to long chain; furthermore, there is no turnover of LPS (13, 14). By growing this mutant for many generations in radioactive galactose, which labels only long chain LPS, and radioactive leucine, which labels protein and not LPS, we can measure the steady-state specific activity (galactose relative to leucine, i.e., LPS/protein) of bacteria completely covered with long chain LPS. It is then possible to calculate the proportion of long chain LPS relative

to total LPS in cells grown continuously in labeled leucine without galactose, and then given labeled galactose for various short lengths of time. LPS is made on the inner membrane and transferred shortly thereafter (within 1-2 minutes) to the outside of the outer membrane (13), so for all times of exposure to galactose except those less than ~ 3 minutes, the percent of labeled long chain LPS present in the organism will accurately reflect the percent of such molecules in the outer membrane. It is therefore possible to prepare Salmonella of known proportions of long and short chain LPS on their surface and to test the effect of these on the fractionation of the bacteria by countercurrent chromatography.

The results of such an experiment are shown in Fig. 2 and Table 1. After several generations of growth in the presence of [^{3}H]leucine and no galactose, [^{14}C]galactose was added and the cells harvested at 0 to 5 minutes thereafter. Table 1 shows the percent of long chain LPS relative to total LPS present on the surface of the bacteria. Figures are not given for 0-3 minutes, since at those early times a large proportion of the LPS is internal, having not been translocated to the outer membrane; however, by 4 minutes more than 85% of all galactose-containing LPS should be in the outer membrane.

When the distribution of such cells on countercurrent chromatography is examined, the following observations were made. As expected, the position of cells within the first 2 minutes of exposure to galactose is identical to that of cells grown without galactose, since little of the new LPS would be on the surface of the cell. However, at the time when a significant proportion of the long chain LPS appears in the outer membrane (~ 2-3 minutes), two phenomena appear. First, heterogeneity appears in peak II, indicating that the presence of some molecules of long chain LPS are affecting the mobility of these cells. Second, a substantial proportion of the cells appear in the position of peak I, even though they differ only slightly in percent long chain LPS from the cells in later fractions. The change is especially striking

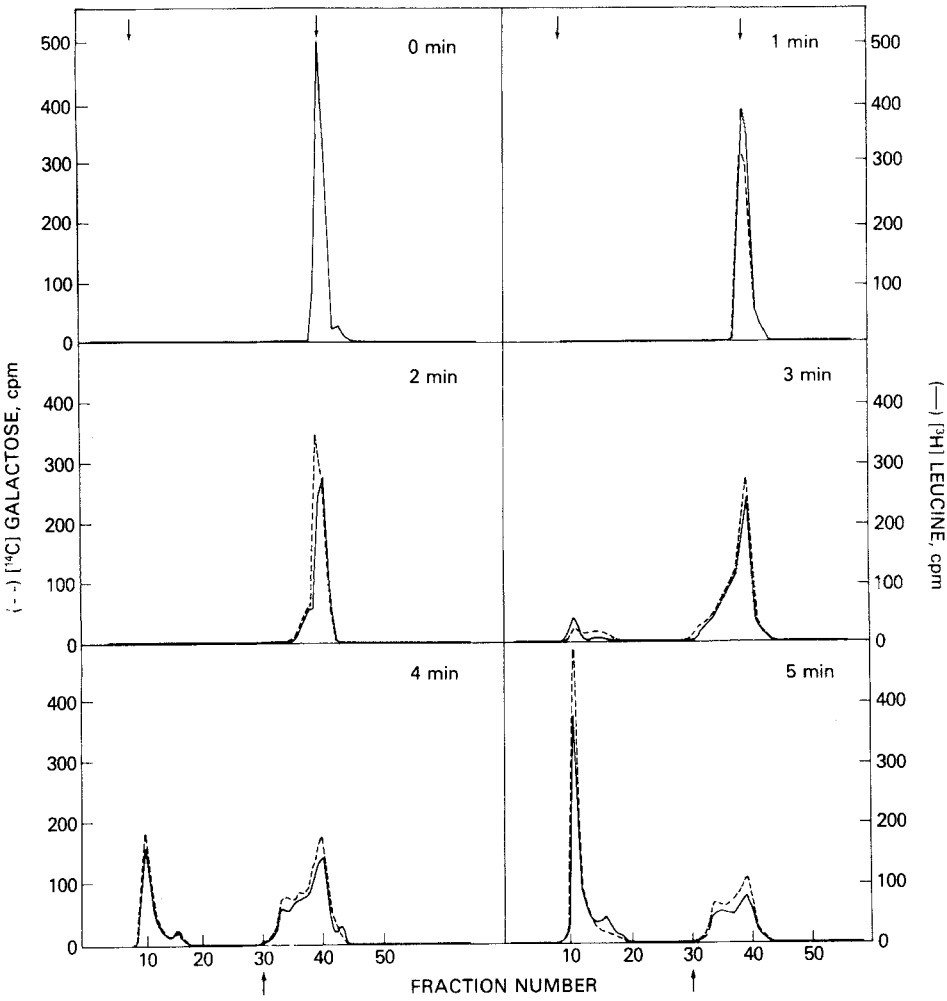

FIGURE 2. Separation of cells of S. typhimurium G30 grown 0-5 minutes with [^{14}C]galactose and continuously with [^{3}H]leucine. Cells were grown without galactose and with [^{3}H]leucine (1 μCi/ml) to a density of 3 x 10^{8}/ml and [^{14}C]galactose added (3 mCi/mmol). Samples were chilled and harvested at 0, 1, 2, 3, 4, and 5 minutes thereafter as described in Fig. 1 and Materials and Methods. The arrows at the top of the figure indicate the position of peaks I (left peak) and peak II (right peak).

TABLE 1

Properties of Cells Grown Varying Short Times in Galactose

Experiment shown in	Duration of exposure to galactose (min)	% Long chain LPS (relative to total) *	% Total cells in †	
			Peak I	Peak II
Figure 2	3	15	7	93
	4	19	31	69
	5	22	63	37
Figure 3	4	31	32	68
	5	35	56	44
	6	36	60	40
	7	39	67	33

*The percent of long chain LPS was calculated as described in the text.

†The percent of total cells in each peak was calculated by dividing the leucine cpm/peak by the total leucine cpm recovered in all fractions.

in comparing the 4-minute to the 5-minute sample: although the total percent of long chain LPS went up only 3% from 19% to 22% (Table 1), the percent of total cells in the area of peak I went from 31% to 63%. No significant difference in percent of long chain LPS is seen in peaks I and II at a given time point. These results suggest that a property other than total percent of long chain LPS affects the partition behavior of these cells in countercurrent chromatography.

To investigate these findings further, the experiment was repeated, except that samples were removed at 4 to 7 minutes after galactose addition (Fig. 3). In this experiment the percent of long chain LPS in the cells increased from 31% to 39% over the time period surveyed. Once again, countercurrent chromatography showed cells falling in three areas: the position of peak I, the position of peak II, and a series of shoulders forming a leading edge to peak II. Once again, there is a dramatic shift in percent of cells falling in peak I between 4 and 5 minutes of

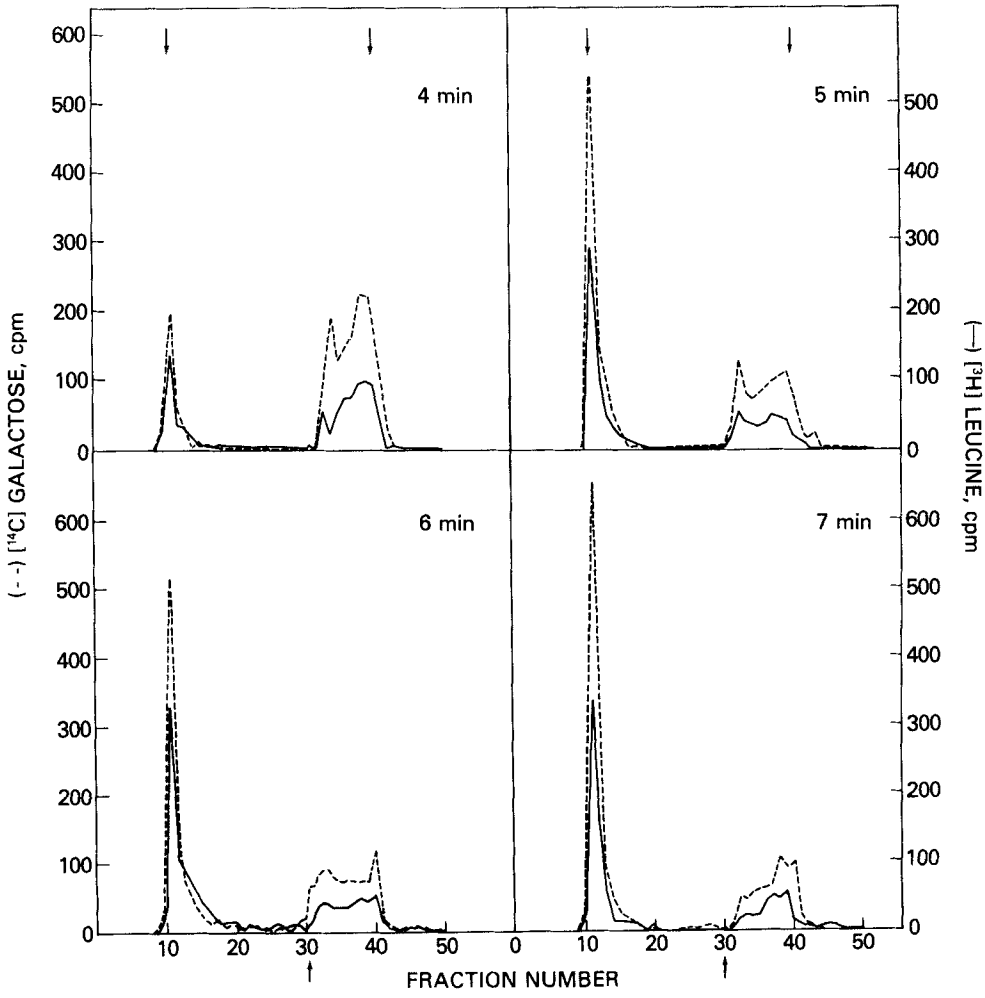

FIGURE 3. Separation of cells of S. typhimurium G30 grown 4-7 minutes with [^{14}C]galactose and continuously with [^{3}H]leucine. Procedure exactly as for Fig. 2 except that cells were harvested at 4, 5, 6, and 7 minutes after addition of galactose.

exposure to galactose (from 32% to 56%), and the percent of cells in peak I continues to increase, somewhat more slowly, over the next few minutes. As with the previous experiment, the percent of long chain LPS present in cells of peak I and peak II at a given time point are substantially the same. In another experiment (data not shown), approximately 90% of cells were in peak I after 11 minutes of exposure to galactose, and 100% in peak I after 20 minutes. These results confirm that some property of the cells other than actual percent of long chain LPS in the outer membrane affects their distribution in countercurrent chromatography.

DISCUSSION

The outer membrane of gram-negative bacteria and the synthesis of its LPS is diagrammed in Fig. 4. LPS is synthesized in the inner membrane (13) and is translocated to the outer membrane at a large number of points, after which it spreads over the surface (14-17). Mutants of S. typhimurium defective in UDP-galactose-4-epimerase provide an excellent tool for studying the effect of changes solely in LPS on the mobility of cells in countercurrent chromatography. When galactose is added to such mutants, the only change in the outer membrane is the addition during subsequent growth of LPS that contains long polysaccharide side chains instead of LPS with short chains. The total proportions of all outer membrane components stay the same. In general, only a tiny percentage of total LPS is found at one time in the inner membrane (perhaps 2%), and translocation of a newly synthesized molecule to the outer membrane is rapid (13, 16), so that at 37°C, 3-4 minutes after addition of labeled galactose to the S. typhimurium mutant, virtually all label will reflect molecules associated with the outer membrane. In contrast, spread of the LPS to cover the surface is much slower, taking 10-20 minutes to reach homogeneity (16-18). It is not known whether spreading of outer membrane molecules occurs from true lateral diffusion or from growth.

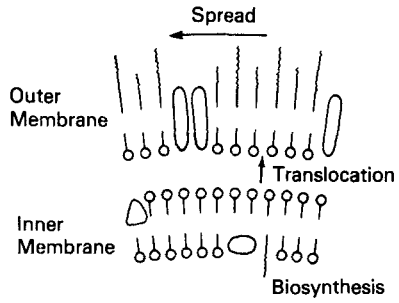

FIGURE 4. Diagram of structure of the outer membrane of S. typhimurium and biosynthesis of LPS. Symbols: = LPS; = phospholipid; = protein. The outer face of the outer membrane is the final destination of LPS, which is synthesized in the inner membrane, translocated to the outer membrane, and spreads to cover the surface. Translocation probably takes place at between 50 and 200 adhesion points between the two membranes (diagrammed by an arrow, but not pictured). It is not known whether spread occurs by diffusion within the bilayer or by growth or both. LPS may appear transiently on the outer face of the inner membrane and/or the inner face of the outer membrane during biosynthesis and translocation (not pictured).

We find with countercurrent chromatography that cells with virtually the same proportion of LPS molecules containing long chain LPS (in the range of 15-40%) can fractionate either in peak I (the position of cells covered entirely with long chain LPS) or in the position of peak II (the position of cells covered entirely with short chain LPS). The percentage of cells that fractionates in each peak changes during the period of 3-11 minutes after addition of galactose to the S. typhimurium G30 mutant. The changes in fractionation pattern do not reflect synthesis of LPS with a different average side chain length immediately after galactose addition, relative to cells grown continuously with galactose, since Palva and Mäkelä (19) showed that the distribution of side chain lengths in LPS was the same at 1 minute after galactose addition as in cells grown continuously with galactose. We can best explain these results by the following hypothesis:

Bacteria with low (15-40%) percentages of long chain LPS in their outer membrane can fractionate either in the position of

cells covered with long chain LPS or cells covered with short chain LPS depending on where the long chain LPS is in the outer membrane. For a few minutes after translocation to the outer membrane the long chain molecules are "hidden", perhaps because they may face inward rather than outward on the outer membrane, or perhaps because they may not have spread over the surface from the points of their insertion. Although we cannot as yet distinguish these hypothesis, it is certain that countercurrent chromatography provides a tool for separating bacteria on the basis of physical properties of their outer membrane that have not been detectable or separable by other means, and thus may be of great help in analyzing steps in membrane biogenesis.

REFERENCES

1. Walter, H. Partition of cells in two-polymer aqueous phases: a surface affinity method for cell separation. In Methods of Cell Separation (Catsimpoolas, N., ed.), Vol. 1, Plenum Press, New York, 1977, p. 307.

2. Walter, H., Krob, E. J., and Brooks, D. E. Membrane surface properties other than charge involved in cell separation by partion in polymer, aqueous two-phase systems. Biochemistry 15, 2959 (1976).

3. Ito, Y. Countercurrent chromatography. J. Biochem. Biophys. Methods 5, 105 (1981).

4. Ito, Y., Bramblett, G. T., Bhatnagar, R., Huberman, M., Leive, L., Cullinane, L. M., and Groves, W. Improved nonsynchronous flowthrough coil planet centrifuge without rotating seals: principle and application. Sep. Sci. Technol. 18, 33 (1983).

5. Lüderitz, O., Freudenberg, M. A., Galanos, C., Lehmann, V., Rietschel, E. H., and Shaw, D. H. Lipopolysaccharides of gram-negative bacteria. In Current Topics in Membranes and Transport. Membrane Lipids of Prokaryotes (Razin, S., and Rottem, S., eds.), Vol. 17, Academic Press, New York, 1982, p. 79.

6. Osborn, M. J., Rosen, S. M., Rothfield, L., and Horecker, B. L. Biosynthesis of lipopolysaccharide, I. Enzymatic incorporation of galactose in a mutant strain of Salmonella. Proc. Natl. Acad. Sci. U.S.A. 48, 1831 (1962).

7. Stendahl, O., Magnusson, K.-E., Tagesson, C., Cunningham, C., and Edebo, L. Characterization of mutants of Salmonella typhimurium by counter-current distribution in an aqueous two-polymer phase system. Infect. Immun. 7, 573 (1973).

8. Magnusson, K.-E., Stendahl, O., Tagesson, C., Edebo, L., and Johansson, G. The tendency of smooth and rough Salmonella typhimurium bacteria and lipopolysaccharide to hydrophobic and ionic interaction, as studied in aqueous polymer two-phase systems. Acta Path. Microbiol. Scand. Sect. B 85, 212 (1977).

9. Stendahl, O., Edebo, L., Magnusson, K.-E., Tagesson, C., and Hjertén, S. Surface-charge characteristics of smooth and rough Salmonella typhimurium bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. Acta Path. Microbiol. Scand. Sect. B 85, 334 (1977).

10. Stendahl, O., Tagesson, C., and Edebo, M. Partition of Salmonella typhimurium in a two-polymer aqueous phase system in relation to liability to phagocytosis. Infect. Immun. 8, 36 (1973).

11. Osborn, M. J., Gander, J. E., and Parisi, E. Mechanism of assembly of the outer membrane of Salmonella typhimurium. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247, 3962 (1972).

12. Nikaido, H. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of gram-negative cell wall. In Bacterial Membranes and Walls (Leive, L., ed.), Vol. 1, Marcel Dekker, New York, 1973, p. 131.

13. Osborn, M. J., Gander, J. E., and Parisi, E. Mechanism of assembly of the outer membrane of Salmonella typhimurium. Site of synthesis of lipopolysaccharide. J. Biol. Chem. 247, 3973 (1972).

14. Muhlradt, P. F., Menzel, J., Golecki, J. R., and Speth, V. Outer membrane of Salmonella. Sites of export of newly synthesized lipopolysaccharide on the bacterial surface. Eur. J. Biochem. 35, 471 (1973).

15. Muhlradt, P. F., Menzel, J., Golecki, J. R., and Speth, V. Laterial mobility and surface sensitivity of lipopolysaccharide in the outer membrane of Salmonella typhimurium. Eur. J. Biochem. 43, 573 (1974).

16. Kulpa, C. F., Jr. and Leive, L. Mode of insertion of lipopolysaccharide into the outer membrane of Escherichia coli. J. Bacteriol. 126, 467 (1976).

17. Bayer, M. E. Role of adhesion zones in bacterial cell surface function and biogenesis. In Membrane Biogenesis (Tszgaloff, A., ed.), Plenum Press, New York, 1975, p. 393.

18. Bayer, M. E. The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. In Bacterial Outer Membranes. Biogenesis and Functions (Inouye, M., ed.), John Wiley and Sons, New York, 1979, p. 167.

19. Palva, E. T., and MäkelÄ, P. H. Lipopolysaccharide heterogeneity in Salmonella typhimurium analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Eur. J. Biochem. 107, 137 (1980).