AN EQUILIBRIUM BETWEEN TWO FRACTIONS OF LIPOPOLYSACCHARIDE IN ESCHERICHIA COLI

By Stuart B. Levy and Loretta Leive

LABORATORY OF BIOCHEMICAL PHARMACOLOGY, NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by Bernard D. Davis, October 2, 1968

When cells of *Escherichia coli* are exposed briefly to ethylenediaminetetraacetate (EDTA), they release approximately half their lipopolysaccharide (LPS).[1, 2] The amount lost for a given strain appears fixed, since it cannot be increased by several changes in procedure, including increase in the time of exposure and concentration of EDTA.[2] Because these results suggested that the released LPS might differ from the remainder, the metabolic relationship between these two fractions was investigated.

Lipopolysaccharide was labeled with galactose-C^{14} for various periods of time, using a mutant that can incorporate this sugar into only LPS, and the amount of labeled LPS released by EDTA treatment was determined. The results show that in the growing cell the fraction of LPS that is released by EDTA is in rapid equilibrium with that which is retained. Newly synthesized LPS is initially part of the retained fraction but rapidly enters this equilibrium.

Materials and Methods.—E. coli J5, a mutant derived from *E. coli* 0111:B4, was used. This mutant lacks UDP-galactose-4-epimerase and cannot form complete LPS without galactose in its growth medium; with galactose, it forms a cell wall having the same structure as that of its parent.[3] This organism was the gift of Dr. Edward Heath.

Cells were grown to mid-exponential phase (5×10^8 cells/ml) in Trypticase soy broth containing 0.25% galactose and 0.25% glucose. Where indicated, LPS was labeled with galactose-1-C^{14} (New England Nuclear Corporation). When labeled cells were diluted into nonradioactive medium and grown for various lengths of time, there was no loss of galactose label from TCA-precipitable material.

EDTA treatment was performed as previously described,[1, 2] except that cells were washed with 0.85% NaCl at 4° before treatment; exposure to EDTA ($2\text{–}6 \times 10^{-4}$ *M*) was terminated after 4–6 min by addition of $MgCl_2$ ($2\text{–}6 \times 10^{-3}$ *M*), and the cells were centrifuged. The amount of LPS in the supernatant and cell-pellet fractions was estimated by assaying for colitose as previously described.[1, 4] This sugar is present only in LPS and its precursors,[5] and its per cent release by EDTA in the parent strain is the same as that of total cell LPS.[2] The amount of labeled galactose in LPS was estimated by precipitating aliquots of the supernatant and cell-pellet material with 5% trichloroacetic acid (TCA) at 4°, filtering on Millipore filters (pore size 0.22 μ), washing once with 2.0 ml cold 5% TCA and three times with 5.0 ml water, and measuring the radioactivity in a scintillation counter as previously described.[1]

In mutants of *Salmonella* lacking UDP-galactose-4-epimerase, virtually all acid-precipitable galactose is in LPS.[6] The same is true for this *E. coli* mutant as shown by the following control experiments: (1) Cells labeled for 2 min and for 3 hr (four generations) were extracted with phenol and the LPS was purified as previously described.[2] In several experiments 65–100% of the original TCA-precipitable counts were recovered in the purified LPS with no systematic differences between the 2-min and the 3-hr sample. For both times of labeling, the ratio of TCA-precipitable galactose-C^{14} to total colitose in the cells was the same as the ratio of total galactose-C^{14} to colitose in the purified LPS. (2) When fully labeled cells were treated with EDTA, the ratio of colitose to TCA-precipitable galactose-C^{14} was the same for the whole cell, the released material, and the material retained in the cells. (3) Highly labeled galactose-1-C^{14} and UDP-galactose-C^{14}

(UL) (New England Nuclear Corp.) were added to unlabeled cells at 0°C. When the cells were precipitated, filtered, and washed under the above conditions, no counts were retained on the filters.

Results.—To determine whether newly synthesized LPS is released by EDTA treatment, radioactive galactose was added to exponentially growing cells, and at various times aliquots were removed and treated with EDTA. As Figure 1 shows, at all times this treatment released half of the total LPS (measured as colitose), but at early times much less labeled LPS was released. Not until approximately 15 minutes after addition of labeled galactose did the per cent release of labeled LPS equal that of total LPS. These experiments indicate that newly synthesized LPS is relatively insensitive to release by EDTA treatment.

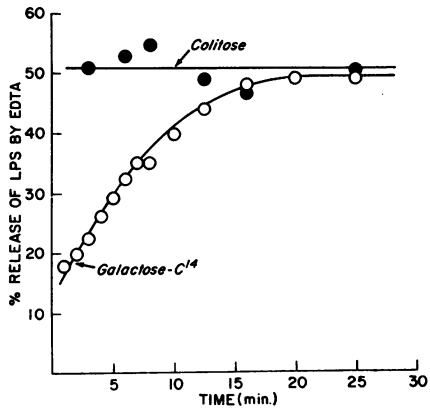

FIG. 1.—Sensitivity of newly synthesized LPS to release by EDTA. At zero time galactose-1-C^{14} (0.3 mc/mM) was added to growing cells of *E. coli* J5 (60 ml). At various times thereafter 5.0-ml aliquots were removed and the cells washed and treated with EDTA. The per cent release of TCA-precipitable galactose-C^{14} and colitose was determined.

To follow the fate of aging LPS, cells were briefly labeled with galactose and then diluted into medium containing unlabeled galactose before being treated with EDTA. Although this dilution terminated entry of new label, incorporation of intracellular TCA-soluble counts into TCA-precipitable material normally continued for 10–15 minutes, presumably owing to the large pool of galactose-1-P and UDP-galactose known to be present in epimeraseless mutants grown in galactose.[7] This time of residual incorporation could be shortened by interposing a period of galactose starvation just prior to labeling. In the experiment shown in Figure 2, cells were deprived of galactose for 8 minutes before being labeled with galactose for 30 seconds. The cells were then diluted into complete medium containing unlabeled galactose, and aliquots were removed at intervals and treated with EDTA. Incorporation of counts into LPS from the acid-soluble pool continued for approximately seven minutes (Fig. 2*a*). In accord with the results shown in Figure 1, at early times labeled LPS was relatively insensitive to release by EDTA; however, the per cent release of labeled LPS increased with time to 50 per cent, equivalent to the release of total LPS (Fig. 2*b*). This maximum was attained at approximately eight minutes, soon after incorporation of labeled precursor was completed. Experiments in which cells were labeled for periods of 30 seconds to 2 minutes without prior starvation and then diluted into unlabeled medium gave essentially the same results, except for a longer pe-

riod of incorporation of label (10–15 min). In all cases, no more than 50 per cent of the counts could ultimately be released by EDTA, and this value was achieved within less than five minutes before the end of incorporation of label into LPS.

The above findings are compatible with either a partitioning of new material into released and retained fractions or an equilibrium between these fractions. Regardless of mechanism, the half time for newly synthesized LPS to reach its final distribution can be determined.[8] This half time was calculated from the experiment depicted in Figure 1 and from three separate experiments like that illustrated in Figure 2, including experiments with and without prior starvation. The four values obtained were 0.92, 1.20, 0.23, and 1.0 minute.

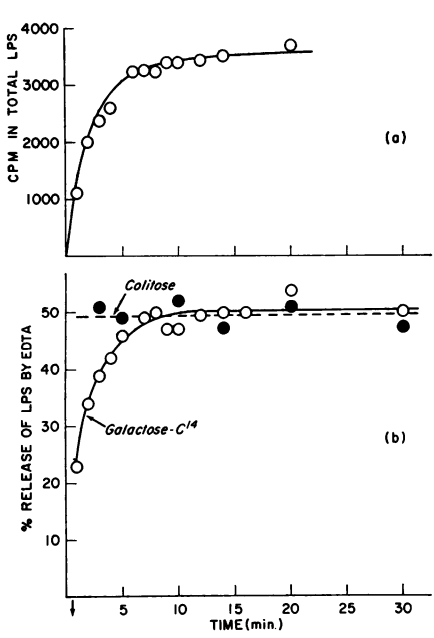

FIG. 2.—Release by EDTA of a 30-sec pulse of labeled galactose after growth in nonradioactive medium. A culture of *E. coli* J5 (50 ml) was centrifuged, resuspended, and incubated for 8 min in the same volume of medium devoid of galactose and glucose. These cells were then centrifuged and resuspended in 1.5 ml of medium containing 0.025% each of glucose and galactose-1-C^{14} (90 mc/mM). Thirty sec later (*b*, *arrow*) the culture was diluted into 155 ml of medium containing 0.25% each of galactose and glucose. At various times thereafter the total amount of TCA-precipitable galactose-C^{14} was measured (*a*). Other samples were treated with EDTA and the per cent release of colitose and TCA-precipitable galactose-C^{14} was determined (*b*).

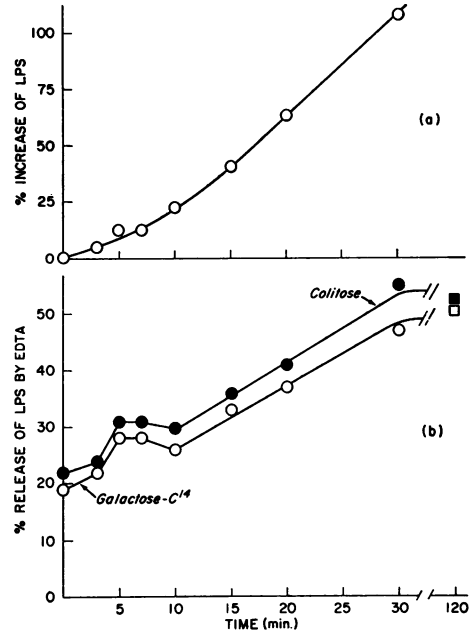

FIG. 3.—Release of previously retained LPS by a second EDTA treatment. A culture of *E. coli* J5 (35 ml) was grown for four generations (160 min) in medium containing galactose-1-C^{14} (0.08 mc/mM) and then for 15 min in unlabeled medium. The cells were subsequently washed, treated with EDTA, and centrifuged, releasing 51% of both colitose and labeled galactose. A portion of the cells was immediately resuspended in prewarmed Tris-Cl and treated with EDTA (*b*, zero time). The remainder was resuspended and incubated in prewarmed complete medium. At various times, aliquots were removed for determination of the per cent increase of LPS as measured by colitose (*a*) and for a second EDTA treatment. The per cent of colitose and TCA-precipitable galactose-C^{14} released by this second treatment was determined (*b*).

In order to determine whether LPS retained on one EDTA treatment will be redistributed during subsequent growth, cells with fully labeled LPS were treated with EDTA (releasing half of the LPS), washed, and allowed to grow in nonradioactive medium. Aliquots were removed at various times and again treated with EDTA (Fig. 3). The zero-time sample shows that this organism released about 10 per cent more of the original LPS (20% of that remaining) when treated for a second time with EDTA.[9] With further growth an increasing fraction of the retained labeled LPS could be released by EDTA; moreover, the fraction of total LPS that could be released increased in an almost identical manner. By 30 minutes the normal maximum release, 50 per cent of the label, was attained. In other experiments, cells grown for more than three generations (120 min) after the first EDTA treatment still released only half of the previously labeled LPS in a second treatment. To ensure that galactose and colitose were measuring equivalent species of LPS during regrowth, the following experiment was performed. Fully labeled and unlabeled cells were grown in parallel and treated with EDTA. The labeled cells were diluted into unlabeled medium, and vice versa. After one generation of growth a second EDTA treatment of each culture released half of the labeled LPS, measured in terms of either galactose or colitose.

Discussion.—These experiments show that newly synthesized LPS is not initially released by EDTA treatment but rapidly achieves an equal distribution between released and retained fractions. The bulk of retained LPS does not appear to be an obligatory precursor of the released fraction (see Figs. 2*b* and 3*b*). The data illustrated in Figures 1 and 2 suggest either a partition of new LPS into released and retained fractions or a rapid exchange of material between these fractions. The data in Figure 3 provide strong evidence for the latter interpretation: as EDTA-treated cells regenerate their LPS, retained labeled LPS shifts into the expanding releasable fraction at a rate that maintains an equal specific activity in the two fractions. It therefore appears that virtually all the LPS of the cell participates in this equilibrium, although it is possible that a small nascent fraction does not. The half time for this equilibrium is approximately one minute.

Robbins[10] has recently obtained very similar results with *Salmonella anatum*. By phenol purification of labeled LPS fractions released and retained after EDTA treatment, he was able to demonstrate that newly synthesized LPS is insensitive to EDTA release and to suggest that an equilibrium exists between the released and retained LPS fractions.

Possibly the released LPS is located externally to the retained fraction, but evidence is still lacking to support this idea. Electron microscopic studies show the *E. coli* surface to consist of two surface layers separated by electron-dense material containing peptidoglycan.[11, 12] Both electron-microscopic[12] and immunologic[13, 14] evidence suggest that some, if not all, of the LPS is in the outer layer. Loss of half the LPS might thus be expected to visibly alter this layer. But in preliminary experiments we,[15] like de Petris,[12] have seen no differences in surface morphology between EDTA-treated and control cells. Morphologic changes do occur when EDTA-treated cells are converted to spheroplasts by the

action of lysozyme; the two layers remain intact, but the material between them is obliterated.[11, 12] Despite this visible change no further LPS is released by such a treatment.[16] Thus it has not yet been possible to demonstrate a spatial difference between the released and the retained LPS fractions. In addition, nascent LPS, which remains with the retained fraction after EDTA treatment, may nevertheless differ in location or in attachment from the bulk of this fraction.

The concept of an equilibrium between two forms of LPS is new to cell-wall physiology. The mechanism is not yet clear. These two LPS fractions may differ in location, conformation, or degree of association with other molecules, such as cations or cell surface macromolecules. Equilibrium between these fractions may involve a change in the LPS molecules themselves or in their molecular environment. Further investigation will be necessary to elucidate the mechanism of this equilibrium and its functional relationship to the cell.

[1] Leive, L., *Biochem. Biophys. Res. Commun.*, **21**, 290 (1965).

[2] Leive, L., S. E. Mergenhagen, and V. K. Shovlin, *J. Biol. Chem.*, in press.

[3] Elbein, A. D., and E. C. Heath, *J. Biol. Chem.*, **240**, 1919 (1965).

[4] Cynkin, M. A., and G. Ashwell, *Nature*, **186**, 155 (1960).

[5] Heath, E. D., and A. D. Elbein, these PROCEEDINGS, **48**, 1209 (1962).

[6] Osborn, M. J., S. M. Rosen, L. Rothfield, and B. L. Horecker, these PROCEEDINGS, **48**, 1831 (1962).

[7] Spyrides, G. J., and H. M. Kalckar, *Biochem. Biophys. Res. Commun.*, **3**, 306 (1960).

[8] We are indebted to Dr. David M. Shames for these calculations. The following model was used: (1) → (2) ⇆ (3), where (1) is the immediate precursor of LPS, (2) is the retained fraction, and (3) is the released fraction. This model was fit to the data by using the Berman-Weiss SAAM digital computer program (Berman, M., and M. F. Weiss, *SAAM Manual* (Washington, D.C.: U.S. Government Printing Office, 1967), USPHS Publication no. 1703). The t 1/2 for equilibration of LPS is then calculated from the relation $0.693\ (k_{3\to 2} + k_{2\to 3})$.

[9] In the parent organism, *E. coli* 0111:B4, no more LPS is released on an immediate second EDTA treatment.[2] The reason for a different finding in J5 is as yet unexplained. Repeated EDTA treatment of J5 (up to four consecutive treatments without any growth) releases 10% of the original LPS on the second treatment and less than 5% on the third and fourth treatments combined.

[10] Robbins, P. W., in *The 27th Symposium of the Society of Developmental Biology*, ed. M. Locke (New York: Academic Press, in press).

[11] Murray, R. G. E., P. Steed, and H. E. Elson, *Can. J. Microbiol.*, **11**, 547 (1965).

[12] de Petris, S., *J. Ultrastruct. Res.*, **19**, 45 (1967).

[13] Mergenhagen, S. E., H. A. Bladen, and K. C. Hsu, *Ann. N.Y. Acad. Sci.*, **133**, 279 (1966).

[14] Shands, J. W., *Ann. N.Y. Acad. Sci.*, **133**, 292 (1966).

[15] Leive, L., and B. Wetzel, unpublished observations; Leive, L., and H. A. Bladen, unpublished observations.

[16] Levy, S. B., and L. Leive, unpublished observations.