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# Analysis of Lipopolysaccharide Biosynthesis in *Salmonella typhimurium* and *Escherichia coli* by Using Agents Which Specifically Block Incorporation of 3-Deoxy-D-*manno*-Octulosonate

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**Antibacterial agents which specifically inhibit CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyltransferase activity were used to block the incorporation of 3-deoxy-D-*manno*-octulosonate (KDO) into lipopolysaccharide. Lipopolysaccharide synthesis ceased, molecules similar in structure to lipid A accumulated, and bacterial growth ceased following addition of such agents to cultures of *Salmonella typhimurium* and *Escherichia coli*. Although four major species of lipid A accumulated in *S. typhimurium*, their kinetics of accumulation were different. The least polar of the major species was $IV_A$ [*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-amino-2-deoxy-α-D-glucose, acylated at positions 2, 3, 2′, and 3′ with β-hydroxymyristoyl groups and bearing phosphates at positions 1 and 4′], a molecule previously isolated from bacteria containing a *kdsA* mutation (C. R. H. Raetz, S. Purcell, M. V. Meyer, N. Qureshi, and K. Takayama, J. Biol. Chem. 260:16080–16088, 1985). Species $IV_A$ accumulated first and to the greatest extent following addition of the inhibitor, with other more polar derivatives appearing only after $IV_A$ attained half its maximal level. In contrast, only two major species of precursor accumulated in *E. coli* following addition of the inhibitor. One of these species was identical to $IV_A$ from *S. typhimurium* on the basis of chemical composition, fast atom bombardment mass spectroscopy, and comigration on Silica Gel H, and it also accumulated prior to a more polar species of related structure. We conclude that the addition of KDO to precursor species $IV_A$ is the major pathway of lipid A-KDO formation in both *S. typhimurium* LT2 and *E. coli* and that accumulation of the more polar species lacking KDO only occurs in response to accumulation of species $IV_A$ following inhibition of the normal pathway.**

The study of the early steps in lipopolysaccharide (LPS) synthesis has been limited to biochemical analysis of mutants blocked in the pathway (1, 7, 12, 15, 20, 22) or direct biochemical analysis of the flow of metabolites in normal bacteria (1, 20, 21). The study of mutants is limited by the lack of direct methods for selecting mutants blocked early in the pathway, while the direct biochemical approach, which itself often begins with the analysis of mutants, can be very difficult because intermediates in the pathway are present in low amounts and subject to rapid turnover (1). The use of specific metabolic inhibitors is a third approach to studying metabolic pathways, one which has only recently been available for the study of LPS synthesis in gram-negative bacteria (5).

In the absence of direct methods for selecting mutants defective in the early steps of LPS synthesis, inventive approaches to enriching for such mutants have been developed (6, 11, 23, 24). These methods have thus far only yielded mutants defective in the 3-deoxy-D-*manno*-octulosonate (KDO) pathway. Such mutants proved invaluable in studying specific aspects of LPS synthesis at the level of KDO metabolism. Inhibition of KDO synthesis or incorporation into LPS leads to the accumulation of molecules which resemble lipid A in structure (7, 16, 23). The biochemical and physiological consequences of such inhibition include (i) accumulation of lipid A derivatives in the inner membrane (15), (ii) growth stasis (24), (iii) translocation of lipid A derivatives to the outer membrane (15), (iv) perturbation of outer membrane structure allowing insertion of the membrane attack complex of complement leading to cell death (R. Goldman and M. Miller, in preparation), and (v) avirulence in a mouse model system (R. Goldman, unpublished data).

A study of early steps in the assembly of LPS in *Escherichia coli* began from the fortuitous isolation of a mutant defective in formation of the basic disaccharide structure of lipid A (14). UDP-*N*-acetylglucosamine is routed to LPS synthesis when it is acylated with β-hydroxymyristic acid by a specific acylase (1) to yield UDP-2,3-diacyl-glucosamine. A portion of UDP-2,3-diacyl-glucosamine is hydrolyzed to yield free 2,3-diacyl-glucosamine, which can be condensed with UDP-2,3-diacyl-glucosamine to yield the basic lipid A disaccharide structure (20), which is then phosphorylated at the 4′ position (21).

We have developed a new class of antibacterial agents which specifically inhibit KDO incorporation into LPS in gram-negative bacteria (5). These new antibacterial agents consist of a KDO analog, α-C-(1,5-anhydro-7-amino-2,7-dideoxy-D-*manno*-heptopyranosyl)carboxylate (II), attached via amide linkage to the C terminus of alanylalanine (Fig. 1). This peptide prodrug (III) is transported by the *opp* permease system, and the inhibitor (II) is released by the action of intracellular peptidases. The free inhibitor then acts as a competitive inhibitor of CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase). The inhibitor (II) was designed on the basis of our studies of the cloned (3), sequenced (4), and purified (9, 10) CMP-KDO synthetase from *E. coli*. In this study, we have used these new antibacterial agents to examine further details of the early events in LPS assembly in *Salmonella typhimurium* and *E. coli*.

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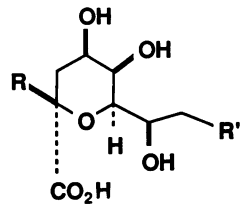


| | R | R' |
|---|---|---|
| I | OH | OH |
| II | H | $NH_2$ |
| III | H | L-ala-L-ala-NH- |

FIG. 1. Structures of KDO (I), CMP-KDO synthetase inhibitor (II), and peptide prodrug III.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *S. typhimurium* strains RG111 (wild type), RG109 (*kdsA50 zdj-3602*::Tn*10*), and RG106 (*kdsB91 zbh-3061*::Tn*10*) and *E. coli* PL2 (*galE relA1*) were from our laboratory collection. Bacteria were grown in MOPS (morpholinepropanesulfonic acid) defined medium (13) containing 0.2% glucose (wt/vol), 1 mM leucine, and 0.5 mM *N*-acetyl-D-glucosamine.

**Antibacterial agents.** Antibacterial agents which specifically inhibit CMP-KDO synthetase were designed and synthesized at Abbott Laboratories as described elsewhere (P. Lartey, D. Riley, R. Hallas, W. Rosenbrook, Jr., D. Norbeck, D. Grampovnik, W. Kohlbrenner, N. Wideburg, and A. Pernet, manuscript in preparation).

**Radiochemical analysis of lipid A derivatives.** Lipid A derivatives were radiolabeled by the addition of (i) *N*-acetyl-D-[1-$^3$H]glucosamine (4 μCi/ml, 0.5 mM final concentration; Amersham Corp. catalog no. TRK.376); (ii) [$^{33}$P]orthophosphoric acid (10 μCi/ml; New England Nuclear Corp. catalog no. NEZ-080); or (iii) [$^3$H]acetate (20 μCi/ml; Amersham catalog no. TRK.12) in MOPS containing 1 mM sodium acetate. Cells were harvested and washed twice with 10 ml of 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer (pH 7.4), and cell pellets were suspended in 1.25 ml of methanol containing 0.1 N HCl; 0.5 ml of $H_2O$ and 0.625 ml of chloroform were then added. The 0.5 ml of $H_2O$ contained 1 mg (dry weight) of RG109 cells which had been shifted to 42°C for 3 h to induce accumulation of lipid A derivatives. Samples were mixed intermittently at room temperature for 20 min, phases were separated by centrifugation at 5,000 × *g* for 5 min, and the lower chloroform phase was saved. Chloroform (1.25 ml) was added to the methanol phase and interface, and the solution was then mixed and centrifuged. The first and second chloroform phases were combined and dried under a stream of $N_2$. The residue was resuspended in chloroform for application to Silica Gel H plates (250 μm; Analtech). Plates were developed in a solvent of chloroform, pyridine, 88% formic acid, and $H_2O$ (40:60:16:5) for 2 h and air dried. Fractions (0.5 cm) were scraped into scintillation vials, and radioactivity was determined after the addition of 100 μl of $H_2O$, followed by 10 ml of Instagel (Packard Instrument Co., Inc.).

The potential degradative effects of extraction on lipid A derivatives from *E. coli* PL2 were examined as follows. Cells treated with III were radiolabeled with *N*-[$^3$H]acetylglucosamine and extracted as described above. A portion of the chloroform phase (0.5 ml) was removed for thin-layer chromatography while the remainder (about 1.5 ml) was evaporated to dryness under nitrogen, and carrier cells (equivalent to 2 ml of cells treated with III, but unlabeled) were added. This cycle was repeated, and the precursor from each cycle was analyzed by thin-layer chromatography.

**Purification of lipid A derivatives.** *S. typhimurium* LT2 strain RG111 and *E. coli* PL2 were grown overnight at 37°C in MOPS plus 0.2% glucose. The next day, 2 to 4 liters of fresh medium containing 0.5 mM *N*-acetylglucosamine was inoculated to an $A_{420}$ of 0.1 and incubated at 37°C in a shaker bath until an $A_{420}$ of 0.4. Peptide prodrug III was added to a final concentration of 50 μg/ml, followed by 50 μCi of *N*-acetyl-D-[1-$^3$H]glucosamine (2.8 Ci/mmol). Incubation was continued at 37°C for 3.5 h. Cultures were harvested by centrifugation, and the pellet was washed twice with cold 50 mM HEPES buffer (pH 7.6). Pellets were stored at −20°C.

Cell pellets were delipidated by a series of extractions with 95% ethanol, followed by acetone and then diethyl ether (22). Delipidated cells were extracted several times in phenol-chloroform-petroleum ether (2:5:8), and the extract was evaporated under nitrogen to the phenol phase. The phenol phase was loaded onto a 2.5 by 11 cm DEAE-cellulose column equilibrated with 99% methanol containing 1% acetic acid. The column was washed with equilibration buffer and then eluted with a linear gradient (500 ml) of 0 to 1 M ammonium acetate in the buffer described above (12, 22). Fractions (4 ml) were collected, and tritium counts for a portion of each fraction were determined with a Packard Tri-Carb 300 scintillation counter. Peak tubes were pooled and extracted into chloroform by using a modified Bligh-Dyer procedure (14, 16).

**Analysis of lipid A derivatives.** The fatty acid contents of chloroform extracts were determined by gas chromatography after conversion to methyl esters with methanolic HCl (2). The mass molecular ion $(M - H)^-$ of lipid A precursors was determined by negative fast atom bombardment mass spectroscopy on a Kratos MS-50 mass spectrometer (AEI/Kratos). Samples in 99% methanol were mixed with a matrix of thioglycerol-glycerol (1:1) or aminoglycerol-glycerol (1:1) on the instrument probe. A neutral beam of xenon was used, and the translation energy varied between 6 and 8 kV. Data were collected at a scan rate of 30 s per decade over a mass range of 160 to 1,600 daltons.

## RESULTS AND DISCUSSION

**Accumulation of lipid A derivatives.** The antibacterial agent III (Fig. 1) causes inhibition of LPS synthesis, inhibition of cell growth, and accumulation of lipid A derivatives in *S. typhimurium* LT2. The four major species which accumulated comigrated with the four major species accumulating when bacteria containing genes coding for temperature-sensitive enzymes of the KDO pathway (*kdsA* or *kdsB*) are shifted to the restrictive temperature (Fig. 2). *E. coli* PL2 ceased growing, and two components similar to those in *S. typhimurium* accumulated after the addition of III (Fig. 2). Both components from *E. coli* were radiolabeled with *N*-[$^3$H]acetylglucosamine, [$^3$H]acetate, and [$^{33}$P]phosphate, indicating that they were the acylated and phosphorylated metabolites of glucosamine. The less polar species comigrated with species $IV_A$, previously isolated and characterized from a *kdsA* strain of *S. typhimurium* (16). The structure of $IV_A$ is *O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-

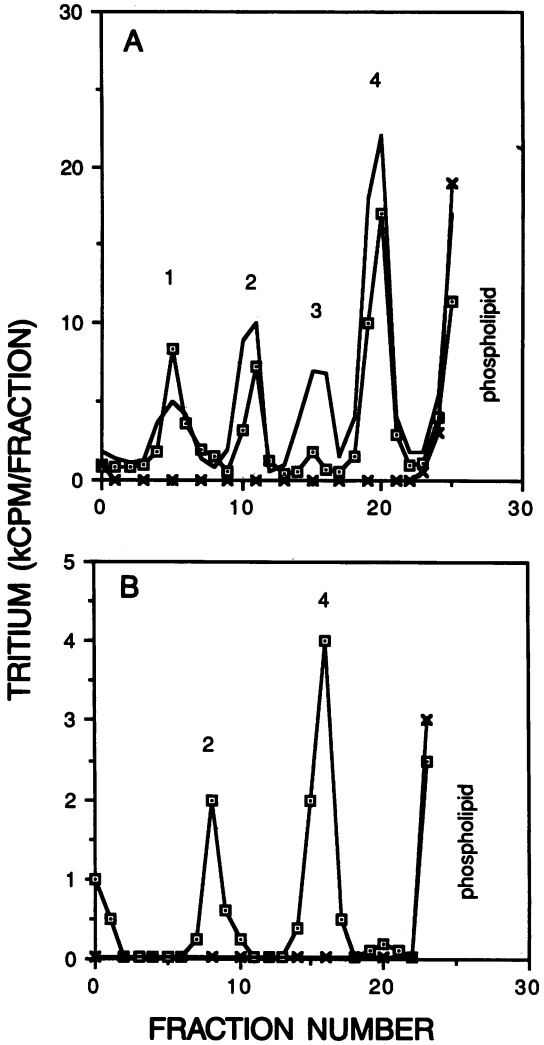


FIG. 2. Analysis of radiolabeled lipid A derivatives. Bacteria were grown in MOPS medium (5 ml) to an $A_{420}$ of 0.2 to 0.3, and III was added to 50 μg/ml simultaneously with *N*-[$^3$H]acetylglucosamine (to 4 μCi/ml). After 3 h, cells were harvested and washed, and lipid A derivatives were extracted into chloroform. Material was fractionated by chromatography on Silica Gel H. Only phospholipid was radiolabeled, because of some dissemination of label, in control cells (no drug), and all new glucosamine-containing metabolites were also radiolabeled with [$^{33}$P]orthophosphoric acid and [$^3$H] acetate. (A) *S. typhimurium* RG111 control (X), 50 μg of III per ml (—), RG106 or RG109 (42°C) (⊡). (B) *E. coli* PL2 control (X), 50 μg of III per ml (⊡). The arabic numerals in panel A denote the four major components from *S. typhimurium*. The arabic numerals in panel B show the relative positions of *S. typhimurium*, components 2 and 4 compared with those from *E. coli* run on the same thin-layer chromatography plate.

amino-2-deoxy-α-D-glucose, acylated at positions 2, 3, 2′, and 3′ with β-hydroxymyristoyl groups and bearing phosphates at positions 1 and 4′ (16). The second more polar species from *E. coli* comigrated with species $III_A$ of *S. typhimurium* (Fig. 2), which is a derivative of $IV_A$ containing phosphoethanolamine attached to the 4′ phosphate. The two other species from a *kdsA* mutant of *S. typhimurium* are designated $I_A$ and $II_A$ (16). Species $I_A$ is identical to $IV_A$ except for the attachment of phosphoethanolamine to the 4′ phosphate and aminopentose to the 1 phosphate (16). Species $II_A$ is identical to $IV_A$ except for the attachment of aminopentose to the 1 phosphate (16). We were concerned that the slower-migrating species from *E. coli* might be a degradation product of the faster-migrating species, since the extraction conditions were acidic. However, the slower-migrating species from *E. coli* is not a degradation product of the faster-migrating species, because the ratio of the two species remained constant during four passages through the extraction procedure (ratios of $IV_A$/slower-migrating species were 1.8, 1.9, and 2.1 for the first, second, and third extraction cycles, respectively).

**Purification and characterization of lipid A derivatives.** Lipid A derivatives that accumulated during treatment with III were purified from both *S. typhimurium* LT2 (strain RG111) and *E. coli* PL2 (Fig. 3). Cells (2 to 3 liters) were grown in MOPS medium and treated with 50 μg of III per ml. Lipid A derivatives were extracted from delipidated cells and purified by chromatography on DEAE-cellulose columns (Fig. 3). Materials designated peaks 1 and 2 (Fig. 3) were pooled for analysis. The molar ratio of glucosamine/β-hydroxymyristic acid/phosphate was 1:2:1 for the peak 2 material isolated from both *S. typhimurium* and *E. coli*, which is in agreement with the compositional analysis of lipid A derivatives isolated from a *kdsA* mutant of *S. typhimurium* (Table 1). Analysis by negative-ion fast atom bombardment mass spectroscopy yielded a major ion $(M - H)^-$ of 1,404 and fragments at 1,178 (loss of a 3-OH-14:0 moiety), 738, 710 (anionic fragment from the reducing end),

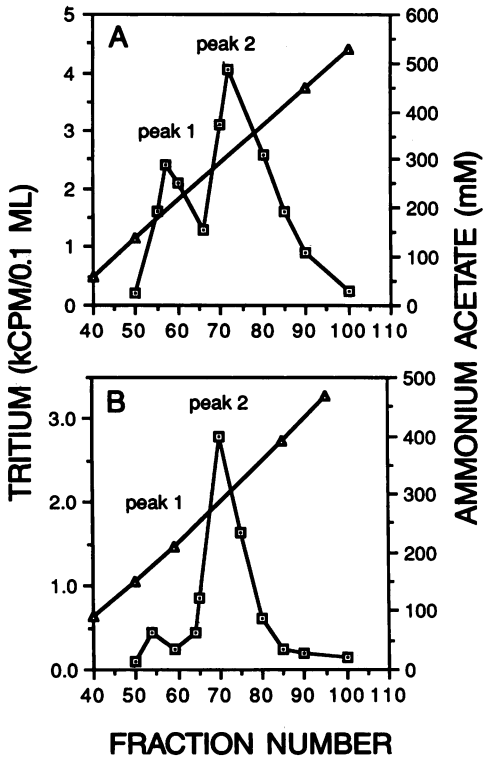


FIG. 3. Purification of lipid A derivatives. *S. typhimurium* RG111 and *E. coli* PL2 were treated with 50 μg of III per ml in MOPS medium containing *N*-[$^3$H]acetylglucosamine for 3 h at 37°C. Lipid A derivatives were extracted, and the final phenol phase was applied to a 2.5 by 11 cm column of DEAE-cellulose. Material was eluted with a gradient of ammonium acetate, and fractions were monitored for radioactivity and conductance. Peak numbers 1 and 2 were collected for further analysis. (A) *S. typhimurium* RG111. (B) *E. coli* PL2. Symbols: ⊡, tritium; △, ammonium acetate.

TABLE 1. Chemical analysis of purified lipid A precursor[a]

| Component | Composition (molar equivalents) | | | |
|---|---|---|---|---|
| | RG111[b] peak | | PL2[b] peak 2 | RG109[c] peak 2 |
| | 1 | 2 | | |
| Phosphate | 3.19 | 1.02 | 1.23 | 0.81 |
| 3-OH-14:0 | 1.26 | 1.80 | 2.05 | 2.21 |

[a] Values were determined in triplicate and were normalized to glucosamine. Glucosamine was present at 1.00 molar equivalent in each peak. KDO was not detectable in any of the peaks.

[b] Precursor accumulation was induced with III, and peaks 1 and 2 from DEAE-cellulose chromatography were analyzed.

[c] RG109 is *kdsA50*. Precursor accumulation was induced by a shift from 30 to 42°C for 3 h.

and 694 $(M—H)^-$ (Fig. 4), all of which are in agreement with the known structure of the major lipid A precursor species $IV_A$ isolated from a *kdsA* mutant of *S. typhimurium* (16). We conclude that treatment of *S. typhimurium* or *E. coli* with III induces the accumulation of lipid A derivatives because CMP-KDO synthetase is inhibited and that the major components in both cases are identical ($IV_A$). Although the less abundant species from *E. coli* (Fig. 2B, fraction 8) contains glucosamine, phosphate, and BHMA and comigrates with species $III_A$ from *S. typhimurium*, we do not know whether they are structurally identical.

Material in peak 1 from DEAE-cellulose chromatography differed between *E. coli* and *S. typhimurium*. Peak 1 from *E. coli* did not contain detectable glucosamine or BHMA but rather contained residual phospholipid (3.9 μmol of $C_{16:0}$, 3.5 μmol of $C_{16:1}$, 4.1 μmol of $C_{18:1}$, 0.33 μmol of $C_{14:0}$, and

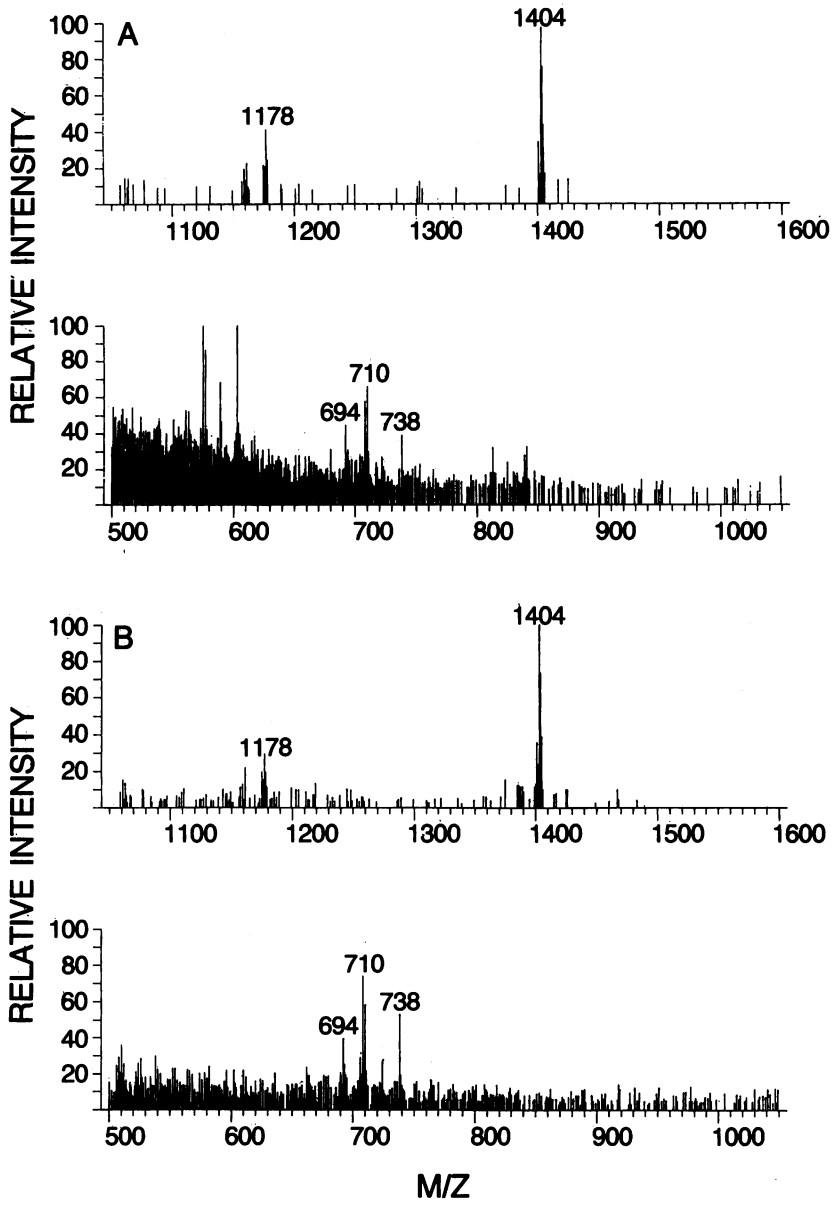


FIG. 4. Fast atom bombardment mass spectrum of DEAE-cellulose peak 2. Purified materials from *S. typhimurium* RG111 (A) and *E. coli* PL2 (B) were analyzed as described in Materials and Methods.

6.2 μmol of phosphate, giving the expected phosphate-to-fatty acid ratio of 0.5). In contrast, peak 1 from *S. typhimurium* did contain glucosamine and BHMA (Table 1), which represented 25% of the total applied. When examined by chromatography on Silica Gel H, peak 1 was enriched for species 1 to 3 and contained residual phospholipid, whereas peak 2 lacked phospholipid but was enriched for species 4 ($IV_A$), which represented 80% of the total material.

**Response of *S. typhimurium* to various doses of III.** The response of *S. typhimurium* LT2 to III was dose related (Fig. 5). The addition of 25 μg/ml or more caused growth stasis, whereas the addition of smaller amounts either transiently affected growth (5 or 10 μg/ml) or showed no effect (1 μg/ml). The accumulation of lipid A derivatives was also dose related, because the addition of 1, 5, 10, 25, 50, or 100 μg of III per ml to 5-ml cultures of RG111 caused the accumulation of 900, 1,800, 3,900, 7,200, 17,100, and 16,500 cpm in lipid A derivatives, respectively, per 200 μg of cell protein.

The transient inhibition of growth at doses below 25 μg/ml is likely due to an increase in the intracellular concentration of free KDO. The inhibitor released from III is an analog of KDO which acts as a competitive inhibitor of CMP-KDO synthetase ($K_i$ = 3 μM). Such inhibition would directly block the utilization of KDO but not its synthesis, a situation which should cause an increase in the intracellular level of KDO. The degree of increase in KDO concentration may be quite extensive, since the enzymes involved in KDO synthesis are apparently insensitive to feedback repression by their reaction products (17, 18, 19). Diazaborine was initially reported to be a specific inhibitor of transfer of KDO from CMP-KDO to lipid A precursor (8); however, later studies

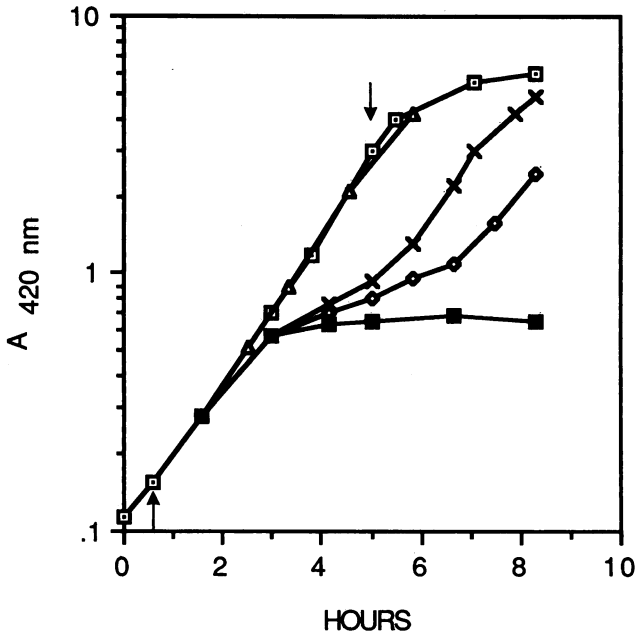


FIG. 5. Dose response of *S. typhimurium* RG111 to III. Cells were grown in MOPS medium, and growth was monitored at $A_{420}$. A single culture was split, various amounts of III were added, along with *N*-[$^3$H]acetylglucosamine, and the effect on cell growth was monitored. III was added at the time indicated by the upward arrow. Cells were harvested at the time indicated by the downward arrow, and lipid A derivatives were extracted and fractionated on Silica Gel H (see text). Symbols: ⊡, control; △, 1 μg/ml; X, 5 μg/ml; ◇, 10 μg/ml; ■, 25, 50, or 100 μg of III per ml, respectively.

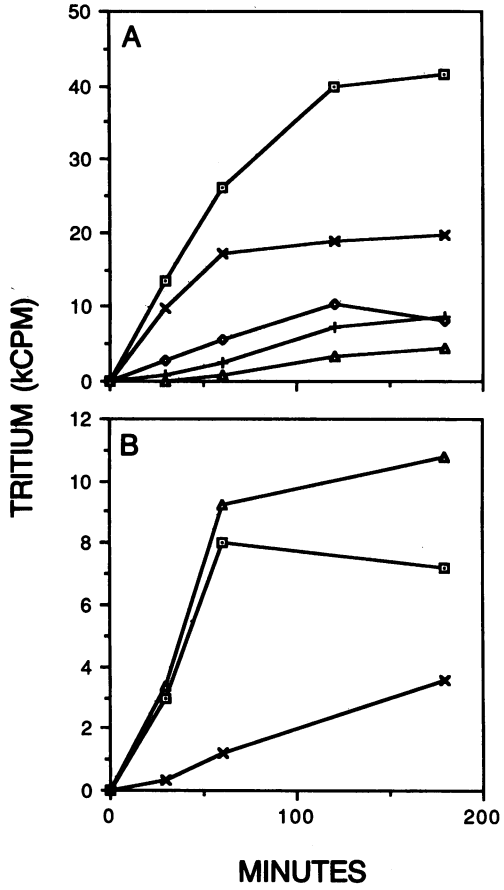


FIG. 6. Kinetics of accumulation of lipid A derivatives. *S. typhimurium* RG111 and *E. coli* PL2 were grown in MOPS medium, and at time 0, III was added to 50 μg/ml, along with *N*-[$^3$H]acetylglucosamine (to 4 μCi/ml). Samples (5 ml) were taken, and lipid A derivatives were extracted and analyzed on Silica Gel H. The relative amount of each precursor species in each 5-ml cell sample was calculated by summing the radioactivity present under each peak. (A) *S. typhimurium* RG111. Symbols: X, $IV_A$; +, $III_A$; ◇, $II_A$; △, $I_A$; ⊡, total $IV_A$ to $I_A$. (B) *E. coli* PL2. Symbols: ⊡, $IV_A$; X, unknown structure; △, total $IV_A$ plus unknown.

(K. Fuchs and G. Hogenauer, Int. Congr. Chemother. 1983, vol. 2, part 88, p. 38–41) suggested that the primary mode of action was inhibition of fatty acid biosynthesis, which then indirectly caused inhibition of lipid A accumulation. We have extended these studies and found that diazaborine inhibits synthesis of all classes of fatty acids (saturated, unsaturated, and hydroxy fatty acids) both in vivo and in vitro when *E. coli* is treated with diazaborine (unpublished data), the likely target being acetyl coenzyme A carboxylase. Interestingly, treatment with diazaborine, which indirectly inhibits LPS synthesis, causes a dramatic increase in the level of free intracellular KDO (8).

**Kinetics of accumulation of lipid A derivatives.** Lipid A derivative $IV_A$ accumulated first and to the greatest amount following addition of III to both *S. typhimurium* and *E. coli* (Fig. 6). The more polar lipid A derivatives from *S. typhimurium* appeared after $IV_A$ and accumulated to a lesser extent, with species $I_A$ appearing last and to the least extent. Similarly, species $IV_A$ was the first to appear in *E. coli*, and it also accumulated to the greatest extent. The more polar

species from *E. coli* appeared after $IV_A$ and accumulated to a lesser extent. It is not a degradation product of $IV_A$ produced during extraction, since multiple retreatments of species $IV_A$ with the standard extraction conditions did not generate the more polar species. Raetz et al. (16) described the pathway leading to $IV_A$ in detail and raised the question of whether species $I_A$ through $IV_A$, or the minor palmitate-containing subspecies $I_B$ through $IV_B$, were intermediates in biosynthesis or side products generated under nonpermissive conditions (i.e., thermal inhibition of the temperature-sensitive *kdsA* gene product). One would expect that the lipid A metabolite which functions as the normal acceptor of KDO would be the first to accumulate following inhibition of KDO synthesis, activation, or transfer to lipid A, after which the metabolic side products would accumulate.

Our data suggest that species $IV_A$ is the normal acceptor for KDO and that the subsequent appearance of the more polar species is due to inhibition of KDO incorporation and accumulation of excess $IV_A$. Since lipid A derivatives translocate to the outer membrane more slowly than LPS and accumulate in the inner membrane (15), it is possible that the enzymes which normally add aminopentose and phosphoethanolamine after KDO addition aberrantly add these polar decorations when $IV_A$ accumulates in the inner membrane. Although we have not identified the precise structure of the more polar species from *E. coli* (it does contain glucosamine, phosphate, and BHMA as the sole fatty acid), the same explanation likely applies to its appearance after $IV_A$ has accumulated. Species $IV_A$ translocates slowly to the outer membrane in *E. coli*, accumulates in the inner membrane, and chases from the inner membrane to LPS following removal of the drug (S. Kadam, manuscript in preparation). Although we have not analyzed its structure, a minor species of a putative lipid A precursor (migrating between $IV_A$ and phospholipid; see Fig. 2B, fraction 20) does appear following prolonged treatment of *E. coli* with the drug. The relationship of this component to $IV_B$ from *S. typhimurium* (16), which also migrates between $IV_A$ and phospholipid, is unknown.

**General conclusions.** The early steps in the assembly of LPS are similar in *S. typhimurium* and *E. coli*. Inhibition of KDO incorporation at the site of CMP-KDO synthetase leads to rapid accumulation of precursor species $IV_A$, followed by a slower and less extensive accumulation of more polar species. Aminopentose has never been reported as a constituent of *E. coli* LPS, and precursor species comigrating with $I_A$ and $II_A$ of *S. typhimurium* (which contain aminopentose) were not observed in the present study. We conclude that species $IV_A$ is likely the normal acceptor of KDO in both *S. typhimurium* and *E. coli* and that the differences in secondary accumulation of more polar precursor species simply reflects species-specific differences in steps which normally occur following the addition of KDO.

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