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Haptenic O-Antigen as a Polymeric Intermediate of *in Vivo* Synthesis of Lipopolysaccharide by *Salmonella typhimurium**

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ABSTRACT: A mutant strain of *Salmonella typhimurium* deficient in phosphomannose isomerase was used to study the kinetics of O-antigen synthesis *in vivo*, these polysaccharides being the sole end products of [^{14}C]-mannose incorporation. The kinetics of uptake of radioactivity into haptenic O-antigen and lipopolysaccharide were consistent with the prediction of an intermediate with high turnover rate. Pulse-chase studies demonstrated rapid and efficient transfer of O-antigenic radioactivity from antigen-carrier lipid hapten to lipopolysaccharide; at least 80% of the label transferred to lipopolysaccharide during the initial chase period was derived from hapten. The addition of completed O-antigenic polymer to the preformed lipopolysaccharide acceptor represents a unique biochemical reaction whereby two different polymers are covalently joined.

Although the role of oligosaccharide intermediates linked to antigen-carrier lipid in biosynthesis of the O-antigen of *Salmonella* is now well established (Weiner *et al.*, 1965; Wright *et al.*, 1965; Osborn and Weiner, 1968), those steps of the pathway which result in attachment of O-antigen chains to lipopolysaccharide are still poorly understood. We have postulated that polymerization of O-antigen chains precedes transfer to lipopolysaccharide, and that the immediate precursor of the O-specific side chains of lipopolysaccharide is an antigen-carrier lipid-linked polysaccharide (O-specific hapten) which occurs as a product of O-antigen synthesis both *in vitro* and *in vivo*. This communication presents evidence in support of the proposed mechanism derived from studies on the kinetics of O-antigen synthesis *in vivo*. For this purpose we have employed a mutant of *Salmonella typhimurium* which lacks phosphomannose isomerase. Under the usual conditions of growth, no

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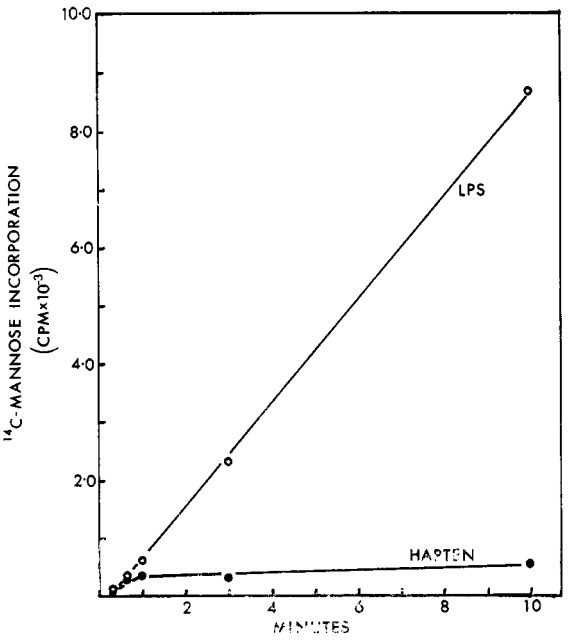

FIGURE 1: Uptake of [^{14}C]mannose into polysaccharides by growing culture of strain *M2* at 22°. [^{14}C]Mannose was added at zero time. The procedures for extraction and separation of labeled components are described under Materials and Methods.

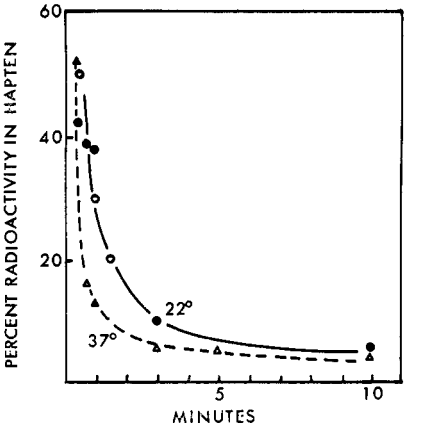

FIGURE 2: Recovery of hapten *vs.* lipopolysaccharide during uptake of [^{14}C]mannose at 22 and 37°. The data summarize the results of two separate experiments at each temperature.

O-antigen is formed because of inability to convert fructose 6-phosphate into mannose 6-phosphate and hence into GDP-mannose. However, synthesis of O-antigen can be initiated by addition of mannose to the culture medium, and under these conditions, O-antigen appears to be the sole end product of mannose metabolism. Measurement of the kinetics of incorporation of [^{14}C]mannose into the O-specific hapten yielded a pattern expected for an intermediate of high turnover rate, and pulse-chase experiments showed a rapid and efficient transfer of radioactivity from the antigen-carrier lipid linked haptenic polymer to lipopolysaccharide.

Materials and Methods

A. Growth on Exogenous Mannose. Strain *M2* of *S. typhimurium*, a mutant deficient in phosphomannose isomerase, was used throughout. Cultures were grown as described previously (Kent and Osborn, 1968a). In midexponential growth, aliquots were transferred to a rotatory shaker bath at 37 or 22°, for 30 min, before addition of 5×10^{-5} M [^{14}C]mannose ($4–10 \times 10^{6}$ cpm/μmole, Schwarz BioResearch, Inc.). At zero time, radioactive mannose was added during rapid stirring of the culture on a magnetic mixer. At intervals thereafter, aliquots were removed to one volume of iced acetone to stop the reaction. The acetone-insoluble material was removed by centrifugation and the pellets were washed twice with cold water. All the radioactivity soluble in 50% acetone is of low molecular weight.

B. Separation of Radioactive Polysaccharides. Difco wild-type lipopolysaccharide (1 mg) and polymeric hapten (2 mg) isolated from strain *SL1032* were added as carrier material. Each sample was then extracted five times for 10 min at 68° with 45% phenol, and the aqueous phases were pooled and counted. Residual phenol was removed by washing the aqueous phase with four volumes of ether; no counts were lost by this procedure. The samples were concentrated to 1.0 ml at 30° under reduced pressure, applied to a column of Sephadex G-50 (1×23 cm), and eluted with 0.02 M NH_4HCO_3; at least 90% of the counts was polymeric for each time point tested (*i.e.*, excluded from the gel), and recovery of label was quantitative. Two volumes of cold ethanol were added to the excluded fractions for selective precipitation of lipopolysaccharides, as previously described (Kent and Osborn, 1968a). Radioactivity was determined with a low-background gas-flow counter.

Results

A. Kinetics of Synthesis of O-Specific Hapten in Vivo. The phosphomannose isomeraseless strain, *M2*, was grown in carbohydrate-free medium; during exponential growth at 37 or 22° [^{14}C]mannose was added and, at indicated intervals thereafter, samples were diluted into one volume of ice-cold acetone. The cells were collected by centrifugation, washed, and extracted with warm aqueous phenol. The radioactivity was quantitatively recovered in the aqueous phase, which contains both the lipopolysaccharide and hapten fractions. The polysaccharides were freed of low molecular weight contaminants by filtration through Sephadex G-50, and the excluded material was separated into lipopolysaccharide and hapten fractions by differential ethanol precipitation. The results are shown in Figures 1 and 2. At both temperatures, detectable incorporation of radioactivity into both lipopolysaccharide and hapten fractions was observed within 20 sec after addition of [^{14}C]-mannose to the cultures. Total mannose incorporation into lipopolysaccharide increased linearly over the period tested; in contrast, the amount of radioactivity in the hapten fraction reached an apparent steady-state level within 1–2 min beyond which time little further increase

was observed. Although it was not possible to determine the true initial rate of [^{14}C]mannose incorporation into hapten from these experiments, the data suggest that the rate of synthesis of the haptenic polysaccharide must be at least equal to that of the O-specific side chains of lipopolysaccharide. Thus, after a 20-sec exposure to [^{14}C]mannose, the hapten accounted for close to 50% of the radioactivity of the total polymer fraction (*i.e.*, lipopolysaccharide plus hapten) (Figure 2). The fraction of polymeric radioactivity present as hapten decreased rapidly with time to a final level of 3 and 5.5% at 37 and 22°, respectively. This is comparable with 3% recovery as hapten when the strain is grown with [^{14}C]-mannose for four to five generations.

Although the hapten accounted for only a small fraction of the total O-specific polysaccharide, the kinetics of [^{14}C]mannose incorporation were consistent with the hypothesis that this material represents a rapidly turning-over precursor of the O-antigen chains of lipopolysaccharide. Direct evidence for this hypothesis was obtained by pulse-chase experiments.

B. Chase of [^{14}C]Mannose-Labeled Hapten into Lipopolysaccharide. In order to demonstrate transfer of radioactive O-specific hapten to lipopolysaccharide, pulse-chase experiments were carried out as follows. A culture of *M2* growing exponentially at 22° in carbohydrate-free medium was exposed to [^{14}C]mannose for 30 sec. At this time an aliquot was removed into ice-cold acetone for analysis, and simultaneously a 4000-fold excess of nonradioactive mannose was added to the remainder of the culture; additional samples were taken over a 9-min period after initiation of the chase (Figure 3). The chase was never completely effective in blocking further incorporation of radioactivity into the total polymer fraction, but this may be related to variable pool size in the several intermediate transformations between mannose and the tetrasaccharide–lipid intermediate from which hapten is formed. However, the data clearly demonstrate rapid turnover of the hapten fraction as well as an efficient chase of the radioactivity initially present as hapten into lipopolysaccharide. Thus, at the end of the 30-sec pulse, approximately 40% of the total [^{14}C]mannose incorporated into polymer was present as hapten. During the first minute of the chase period, the radioactivity of the hapten fraction decreased by 80%, while that of the lipopolysaccharide fraction continued to increase in linear fashion. Although a small amount of additional radioactivity was incorporated into polymer during this time, this amount could not account for the observed increase of label in lipopolysaccharide. In the first minute of the chase, counts recovered as hapten decreased from 3785 to 450 cpm (*i.e.*, a loss of 3335 cpm) while those in lipopolysaccharide increased from 5085 to 9932 cpm (*i.e.*, an accretion of 4847 or 1512 cpm more than lost from the hapten). Thus at least 80% of the radioactivity incorporated into lipopolysaccharide during this time was derived from hapten.

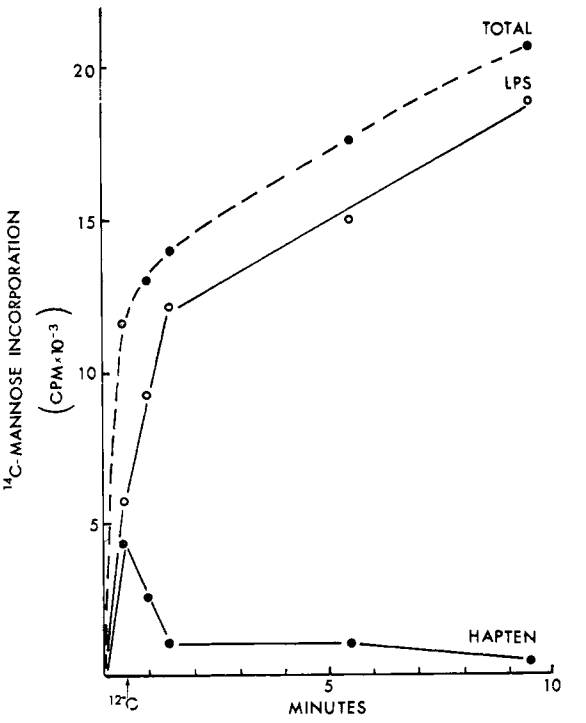

FIGURE 3: Pulse and chase of [^{14}C]mannose into polymeric form by exponentially growing *M2*. Exponentially growing *M2* (65 ml) was pulsed with 5×10^{-5} M [^{14}C]mannose at 91 μCi/μmole. After 30 sec, an aliquot of 12 ml was removed to one volume of iced acetone, unlabeled mannose was added to 2×10^{-2} M (4000-fold excess), and incubation was continued. An aliquot of equal volume was removed at each time indicated, and the radioactive components were extracted as described under Materials and Methods.

Discussion

The postulated addition of O-specific side chains to lipopolysaccharide by transfer of completed polymer chains from the antigen-carrier lipid to the lipopolysaccharide core represents a unique reaction whereby two different polymers are covalently joined after each has been synthesized by a different biosynthetic mechanism, the lipopolysaccharide acceptor by sequential addition of individual hexose residues (Osborn *et al.*, 1964) and the O-specific hapten by polymerization of tetrasaccharide repeating units from a lipid-bound intermediate. The postulated transfer of *polymeric* O-specific material was based on observations that enzymatic polymerization of O-antigen in isolated cell envelope fractions yielded chains which remain attached to the intermediate carrier lipid, and that a similar product (O-specific hapten) is accumulated by mutants which form incomplete core structures. However, direct evidence of the postulated transfer reaction was lacking and other findings suggested that elongation of O-specific side chains might occur by successive transfer of individual repeating units from the tetrasaccharide–lipid intermediate directly to lipopolysaccharide. These included the demonstration of enzymatic transfer of a single, incomplete repeating unit (rhamnosylgalactose) to lipopolysaccharide (Nikaido, 1965), and the identification of a class of mutants (semirough type C) whose lipopolysaccharide contains abortive O-specific chains composed of single tetrasaccharide units (Naide *et al.*,

1965). These data refer only to the first repeating unit attached to lipopolysaccharide. Moreover, the genetic studies of Mäkelä (1966) on such semirough forms suggest the existence of two unlinked loci, one involving genes governing addition of the first repeating unit and the second controlling the presence or the absence of long side chains attached onto the first unit.

The results reported here demonstrate, in intact cells, efficient transfer of O-specific polymer to acceptor lipopolysaccharide, and thereby show that the polymer linked to antigen-carrier lipid can be a normal intermediate in O-antigen synthesis. These experiments do not exclude the possibility of concomitant transfer of individual repeating units to lipopolysaccharide during chain elongation, but the recent demonstration by Robbins *et al.* (1967), that growth of the lipid-linked polymer takes place at its reducing end, renders this unlikely. Further clarification of detailed mechanisms of the transfer and the specificity of the enzyme(s) should emerge from studies on solubilized and isolated enzyme systems in which the relevent steps can be studied individually.

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