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# Biosynthesis of Cell Wall Polysaccharide in Mutant Strains of *Salmonella*. III. Transfer of L-Rhamnose and D-Galactose*

Hiroshi Nikaido

ABSTRACT: A rough mutant of *Salmonella typhimurium*, TV 208, originally isolated by Stocker and Subbaiah [T. V. Subbaiah and B. A. D. Stocker, *Nature 201*, 1298 (1964)], lacks enzyme(s) converting thymidine diphosphate 4-keto-6-deoxy-D-glucose into thymidine diphosphate L-rhamnose (TDPrhamnose). Because of this defect it synthesizes an incomplete cell wall lipopolysaccharide of rough, or, more precisely, $R_{II}$ type. When a particulate fraction from the sonic extract of this strain was incubated with [$^{14}$C]TDPrhamnose, the sugar was transferred to the incomplete lipopolysaccharide contained in the particulate fraction. The incorporation was greatly stimulated by adding nonradioactive uridine diphosphate D-galactose (UDPGal), and nonradioactive TDPrhamnose in turn stimulated the incorporation of [$^{14}$C]D-galactose from UDPGal. However, the addition of guanosine diphosphate D-mannose had little effect on the incorporation of either [$^{14}$C]L-rhamnose or [$^{14}$C]D-galactose. Periodate oxidation showed that all the rhamnose incorporated in the presence of UDPGal occupied a nonreducing terminal position. Partial acid hydrolysis of lipopolysaccharide containing the incorporated galactose and rhamnose yielded an oligosaccharide which appeared to be rhamnosylgalactose. From these results we propose that D-galactose is first attached to the incomplete lipopolysaccharide of $R_{II}$ type, then followed by the transfer of L-rhamnose. These reactions are believed to represent steps in the biosynthesis of the complete lipopolysaccharide produced by smooth strains.

Much work has already been done on the chemical composition of the cell wall lipopolysaccharides (LPS)[1] from enteric bacteria, such as *Salmonella*. When LPS is treated with acid, it is dissociated into a lipid (lipid A) and a water-soluble polysaccharide (Westphal and Lüderitz, 1954). The polysaccharides obtained from the LPS of many *Salmonella* species are often very complex in composition; for instance, the one from *Salmonella typhimurium* would contain an aldoheptose, phosphate, D-glucose, D-mannose, D-galactose, L-rhamnose, abequose (Kauffmann *et al.*, 1960; Krüger *et al.*, 1962), 2-keto-3-deoxyoctonate (Heath and Ghalambor, 1963; Osborn, 1963), and *N*-acetyl-D-glucosamine (I. W. Sutherland, O. Lüderitz, and O. Westphal, to be published; Osborn *et al.*, 1964).

The biosynthesis of these complex polysaccharides, as that of less complicated polysaccharides, probably involves two distinct stages: first, the "activated" form of each constituent sugar is synthesized, usually as nucleoside diphospho sugar (Leloir, 1953), and then the activated sugars are transferred to the acceptor molecule. If a mutation results in the loss of ability to make an "activated" sugar, the cell would synthesize the cell wall polysaccharide up to the point where the sugar in question would be normally transferred, and then the synthesis would stop there, leaving an incomplete polysaccharide.

Using UDPGal-4-epimerase-less mutants of *Salmonella enteritidis* and *S. typhimurium*, which cannot make activated D-galactose (UDPGal), we have shown that they indeed synthesized an incomplete polysaccharide containing only heptose, glucose, and phosphate (Fukasawa and Nikaido, 1960; Nikaido, 1961; Nikaido, 1962a). These results indicated that the central "core" of cell wall polysaccharides from these *Salmonellae* consists of these three components, that D-galactose is then transferred onto this core, and that L-rhamnose, D-mannose, and abequose are transferred later than the galactose moiety in question. The postu-

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[1] Abbreviations used: LPS, lipopolysaccharide; UDPGal, uridine diphosphate D-galactose; UDPG, uridine diphosphate D-glucose; TDPrhamnose, thymidine diphosphate L-rhamnose; TDPG, thymidine diphosphate D-glucose.

lated transfer of D-galactose from UDPGal onto the mutant polysaccharide was actually demonstrated using a cell-free system prepared from these mutants (Nikaido, 1962b; Osborn *et al.*, 1962).

It is obvious that mutants blocked in the synthesis of various other activated sugars besides D-galactose would be of great value in the study of biosynthesis of LPS. In fact, such mutants were found and analyzed recently: those blocked in the synthesis of activated D-glucose (UDPG) (Fukasawa *et al.*, 1962; Sundararajan *et al.*, 1962; Fraenkel *et al.*, 1963) or of activated D-mannose (guanosine diphosphate mannose) (Osborn *et al.*, 1964). This paper describes the characteristics of another such mutant, strain TV 208, which was shown to be unable to synthesize activated L-rhamnose (TDPrhamnose) (Nikaido *et al.*, 1964).

## Materials and Methods

*Bacterial Strain.* TV 208 was isolated as a rough mutant of *S. typhimurium* LT2 by Subbaiah and Stocker (1964). Beckmann *et al.* (1964b) have shown that the strain produced a typical "rough"-type LPS containing only glucosamine, heptose, glucose, and galactose as constituent sugars, with $R_{II}$ immunological specificity.

The mutant frequently gave rise to smooth revertants. Therefore, each inoculation was made from a single colony whose purity was checked by growing the cells in broth without agitation and looking for heavy deposit of the cells.

*Preparation of Cell-free Extract.* The cells were grown overnight in Difco nutrient broth at 37°. The culture was then diluted 1:10 with fresh broth and was grown for 210 minutes at 37° with rotary shaking (240 rpm). The culture was chilled and all the subsequent operations were carried out at 0–4°. The cells were harvested by centrifugation, washed once with water, and suspended in Tris-$MgCl_2$-EDTA (0.05–0.01–0.001 M, *p*H 7.5) (5 ml for the cells from 1 liter culture). The suspension was sonicated with a Raytheon 10 kc oscillator for 3.5 minutes, and the sonicate was centrifuged at 1100 × *g* for 10 minutes. The supernatant was centrifuged at 18,000 × *g* for 20 minutes, and the sediment was taken up in a small amount of 0.005 M Tris-buffer, *p*H 7.5. This was used as "enzyme" or "particulate fraction" in most of the experiments. In some cases, "enzyme" was washed by suspension in 40–50 ml 0.05 M Tris-HCl buffer, *p*H 7.5, and centrifugation at 18,000 × *g* for 20 minutes. This preparation was called "washed enzyme" or "washed particulate fraction."

*Preparation of TDPrhamnose.* For the preparation of TDP-[1-$^{14}$C]rhamnose, [1-$^{14}$C]D-glucose 6-phosphate was enzymatically converted into TDPG in the presence of the crude extract of *Pseudomonas aeruginosa* ATCC 7700, phosphoglucomutase, thymidine triphosphate, $MgCl_2$, and Tris buffer (Kornfeld and Glaser, 1961). After the reaction was stopped by heating at 100°, TDPG was enzymatically converted into TDP-rhamnose essentially as described by Glaser and Kornfeld (1961). The product was purified by adsorption to and elution from charcoal, followed by paper chromatography with the neutral solvent of Paladini and Leloir (1952), where TDPrhamnose moved slightly ahead of TDPG.

TDPrhamnose labeled uniformly with $^{14}$C in the rhamnose moiety was made by the same method except for the use of [U-$^{14}$C]D-glucose 6-phosphate, which was prepared from uniformly labeled [$^{14}$C]D-glucose by the action of hexokinase and ATP.

Nonradioactive TDPrhamnose was prepared similarly, except that the first incubation step was omitted and that TDPG was used as starting material.

*Other Chemicals.* UDPGal and [$^{14}$C]UDPGal (labeled in the galactose moiety) were prepared as described previously (Morikawa *et al.*, 1964; Nikaido, 1962b). [$^{14}$C]UDPG-Gal is the product purchased as [$^{14}$C]-UDPGal from International Chemical and Nuclear Corp. Enzymatic assay (Maxwell, 1957) showed that the material was a mixture of 65% UDPG and 35% UDP-Gal. Anti-$R_{II}$ serum, prepared by immunizing rabbits with another rough mutant of *S. typhimurium* producing $R_{II}$-type LPS, was kindly given by Dr. B.A.D. Stocker.

The following chemicals were obtained from commercial sources: TDPG and thymidine triphosphate (Calbiochem); hexokinase, ATP, and UDPG (Sigma); [1-$^{14}$C]D-glucose 6-phosphate and [$^{14}$C]D-glucose (uniformly labeled) (New England Nuclear Corp.); phosphoglucomutase (Boehringer).

*Typical Procedure for Incorporation Experiment.* The incubation mixture contained enzyme; Tris-maleate buffer, *p*H 6.5, 10 μmoles; $MnCl_2$, 0.5 μmole; and [$^{14}$C]-TDPrhamnose, 5.4 mμmoles containing 7.2 × $10^3$ cpm; in a total volume of 0.2 ml. The reaction was stopped by adding 5% trichloroacetic acid. This acid precipitated the LPS which is probably associated with the fragments of cell wall and/or cell membrane, and the incorporation into LPS could thus conveniently be measured by determining the radioactivity of the cold trichloroacetic acid–insoluble fraction, as was originally shown by Osborn *et al.* (1962). After being washed four times by centrifugation and resuspension in cold 5% trichloroacetic acid, the precipitate was taken up in dilute $NH_4OH$, and aliquots were plated and counted on a Nuclear-Chicago low-background gas-flow counter with Micromil window (background counting rate, 1–2 cpm). Only 2–5 cpm was usually detected in 0-time samples, indicating the almost complete removal of the radioactive substrate.

*Paper Chromatography.* The following solvents were used: A, 1-butanol-pyridine-water (6:4:3) (Jeanes *et al.*, 1951); B, phenol-water (80:20); C, ethyl acetate–acetic acid–water (12:3:3) (Smith, 1958); D, 1-butanol–acetic acid–water (4:1:5, upper phase).

*Thin-Layer Chromatography.* Thin layer of cellulose (MN 300) was used (Randerath, 1962). Solvents were the same as those for paper chromatography.

*Paper Electrophoresis.* This was performed on a sheet of Whatman 3MM paper using the following buffer systems. A, pyridine–acetic acid–water, *p*H 3.5 (1:10:389); B, 0.05 M sodium borate, *p*H 9.2; C, 0.085 M so-

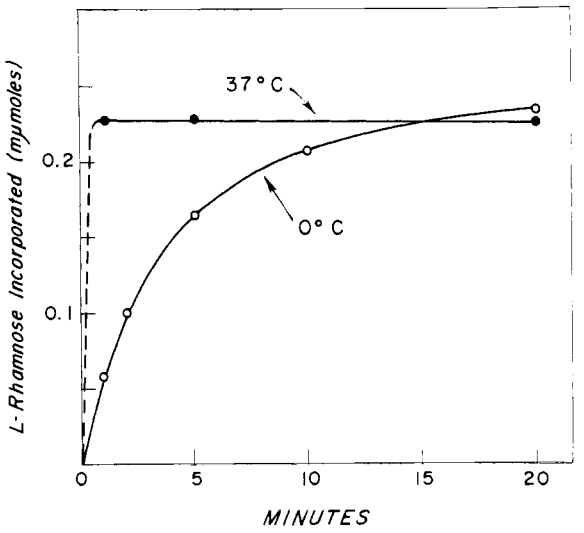


FIGURE 1: Incorporation of [$^{14}$C]rhamnose from [$^{14}$C]TDPrhamnose into trichloroacetic acid-insoluble fraction. The system contained sodium phosphate buffer, *p*H 7.5, 20 μmoles; [$^{14}$C]TDPrhamnose (specific radioactivity 1.33 × $10^6$ cpm/μmole), 5.4 mμmoles; and "enzyme" from TV 208 (prepared as described in Methods), 0.6 mg protein, in a total volume of 0.2 ml. Incorporation was measured as described in Methods.

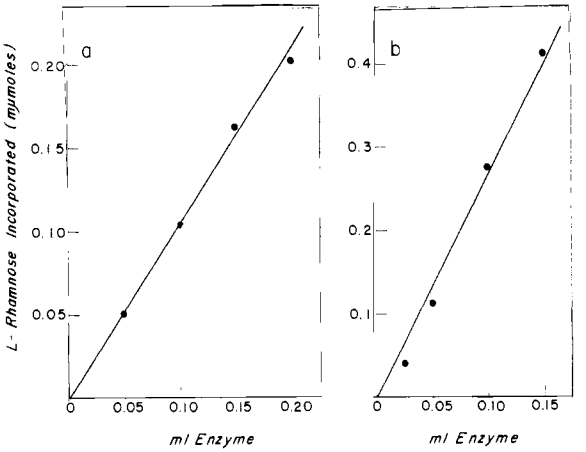


FIGURE 2: Proportionality of the rate of incorporation (a) and the maximum amount of incorporation (b) to the amount of enzyme added. The system contained, in a total volume of 0.25 ml, Tris-maleate buffer, *p*H 6.5, 12.5 μmoles; [$^{14}$C]TDPrhamnose (specific radioactivity 1.33 × $10^6$ cpm/μmole), 5.4 mμmoles; $MnCl_2$, 0.5 μmole; and various amounts of "enzyme" from TV 208 (10.5 mg protein/ml). The reaction was stopped either after two minutes at 0° (a) or after 15 minutes at 37° (b). The preparation of "enzyme" and the measurement of incorporation are described in Methods.

dium molybdate, *p*H adjusted to 5.0 with $H_2SO_4$ (Bourne *et al.*, 1960); D, 0.1 M sodium arsenite, *p*H 9.6 (Frahn and Mills, 1960).

*Periodate Oxidation and the Isolation of Formic Acid* (see Dyer, 1956). The sample was oxidized with 0.03 M (final concentration) periodate at *p*H 5 for 72 hours in the dark (4°) in the presence of 50 μmoles of formic acid as carrier. Excess periodate was destroyed with ethylene glycol, and iodate was removed by the addition of excess $Ba(OH)_2$. The alkaline supernatant was taken to dryness *in vacuo* to remove volatile aldehydes, and the residue was dissolved in water and was dried again. The residue was then dissolved in water, *p*H was adjusted to 2 with phosphoric acid, and the solution was distilled to dryness *in vacuo* into an ice-cooled receiver. The distillation was repeated twice more, each time with addition of 10 ml. of water. The distillates, which would contain any formic acid released, were combined, neutralized with $Ba(OH)_2$, and dried *in vacuo*, and the dried residue was dissolved with a known amount of water. Aliquots of this preparation and of the original sample were counted in a Packard Tri-Carb liquid scintillation counter, using a heterogeneous counting system (Gordon and Wolfe, 1960).

## Results

*Properties of the Mutant Strain.* Strain TV 208 is a rough mutant of *S. typhimurium*. In contrast to the smooth wild type, its LPS was shown by Beckmann *et al.* (1964b) to be entirely lacking in mannose, rhamnose, and abequose.

We examined whether this strain was capable of making various nucleotide-sugars necessary for the biosynthesis of smooth-type LPS (Nikaido *et al.*, 1964). It was known that TDPrhamnose could be isolated from various organisms including enteric bacteria (Okazaki, 1960; Baddiley and Blumson, 1960), and that TDPG was converted enzymatically into TDPrhamnose through TDP-4-keto-6-deoxyglucose as an intermediate (Glaser and Kornfeld, 1961; Pazur and Shuey, 1961; Okazaki *et al.*, 1963). The crude extract of wild type *S. typhimurium* (strain LT2) also catalyzed this reaction, while extracts of mutant TV 208 could not convert TDP-4-keto-6-deoxyglucose into TDPrhamnose, although they could catalyze the reactions presumed to be necessary for the synthesis of various other nucleotide sugars (Nikaido *et al.*, 1964). As a result of this defect, this mutant accumulated large amounts of TDP-4-keto-6-deoxyglucose; when TV 208 cells grown in broth were extracted with hot 70% ethanol and the extract was chromatographed on a column of Dowex 1 (chloride) using a gradient elution with LiCl, 7.7 μmoles of TDP-4-keto-6-deoxyglucose was obtained from 10 g (dry weight) of cells. The same enzymic defect has already been reported in *Escherichia coli* strain Y-10 (Okazaki *et al.*, 1963).

Since mutant TV 208 frequently gave rise to smooth revertants, which could convert TDPG into TDPrhamnose as readily as the parent smooth strain, it could be assumed that the roughness of the strain is the result of its failure to make TDPrhamnose.

*Incorporation of Radioactivity from* [$^{14}$C]*TDPrham-*

*nose*. When the particulate fraction from the sonic extract of this strain was incubated in the presence of [$^{14}$C]TDPrhamnose, the radioactivity was rapidly incorporated into trichloroacetic acid–insoluble material. As Figure 1 shows, the reaction reaches an end point very early at 37° in less than 1 minute, and even at 0° it was almost complete after 10 minutes. Therefore, the *rate* of the transfer reaction was measured by incubating the reaction mixture at 0° usually for 2 minutes.

*Properties of the Incorporation System.* Both the rate of incorporation and the saturation level of incorporation (measured after 15-minutes incubation at 37°) were proportional to the amount of "enzyme" used (Figure 2). The *p*H optimum was around 6.5, using Tris-maleate and Tris-phosphate buffers.

Metal requirement was tested in Tris-maleate buffer, *p*H 6.5 (Table I). $Mn^{2+}$ at 1–5 × $10^{-3}$ M enhanced the rate of reaction. The degree of stimulation was different with the particular enzyme preparations used but was usually in the range of 60–140% over the control. $Co^{2+}$ and $Fe^{2+}$ stimulated approximately to the same extent as $Mn^{2+}$. $Ni^{2+}$, $Zn^{2+}$, and $Ca^{2+}$ were inactive. $Mg^{2+}$ was also without effect at 2.5–10 × $10^{-3}$ M. $K_M$ for TDPrhamnose was 2.3 × $10^{-6}$ M, as determined by the Lineweaver-Burk plot (Figure 3).

The saturation level could not be increased by change of *p*H, increase of substrate concentration, or addition of metal ions, but was markedly increased by the addition of nonradioactive UDPG or UDPGal. This phenomenon will be discussed below. The "enzyme" was stable for weeks when kept frozen at −90°.

It should be noted that the particulate fraction contained both the transglycosylase and the glycosyl acceptor. The addition of purified LPS from the same strain was without effect on the reaction. A similar observation was made earlier with the galactosyl transferase of UDPGal-4-epimerase-less mutants (Osborn *et al.*, 1962; H. Nikaido, unpublished).

The strain specificity of the reaction is shown in Table II. It is clear that [$^{14}$C]rhamnose was not incorporated into the trichloroacetic acid–insoluble fraction by the particulate preparation of those strains which could synthesize TDPrhamnose. These results may be explained as follows. The particulate fractions from LT2 (smooth) and TV 160 (rough, *rouA*) may not contain enough of the proper acceptor LPS, because LPS in the former strain may have terminal sugar units to which rhamnose cannot be attached directly,[2] and the LPS in the latter strain is too incomplete and lacks the terminal unit where rhamnose can be attached (Nikaido *et al.*, 1964). The particulate fraction from TV 119 (rough, *rouB*) may lack the rhamnosyl transferase or the enzyme(s) catalyzing the transfer of

TABLE I: Effect of Divalent Cations on the Incorporation of [$^{14}$C]Rhamnose.[a]

| Expt | Cations Added | Concentration (M) | Rate of Incorporation[b] |
|---|---|---|---|
| I | $Mn^{2+}$ | $10^{-3}$ | 222 |
| | | 2.5 × $10^{-3}$ | 229 |
| | | 5 × $10^{-3}$ | 237 |
| | $Mg^{2+}$ | 2.5 × $10^{-3}$ | 107 |
| | | $10^{-2}$ | 105 |
| II | $Mn^{2+}$ | 2.5 × $10^{-3}$ | 157 |
| | $Co^{2+}$ | 2.5 × $10^{-3}$ | 157 |
| | $Fe^{2+}$ | 2.5 × $10^{-3}$ | 180 |
| | $Ni^{2+}$ | 2.5 × $10^{-3}$ | 96 |
| | $Zn^{2+}$ | 2.5 × $10^{-3}$ | 81 |
| | $Ca^{2+}$ | 2.5 × $10^{-3}$ | 86 |

[a] The incubation mixture contained, in a total volume of 0.2 ml, Tris-maleate buffer, *p*H 6.5, 10 μmoles; [$^{14}$C]TDPrhamnose, 2.7 mμmoles (specific activity 1.33 × $10^6$ cpm/μmole); metal ions (added as chlorides) as indicated; and "enzyme" from TV 208 (1.1 mg protein) previously washed extensively in Tris-HCl buffer by centrifugation and resuspension. Incubation was for 2 minutes at 0°. [b] Incorporation in control tubes without any added metal ion was taken as 100.

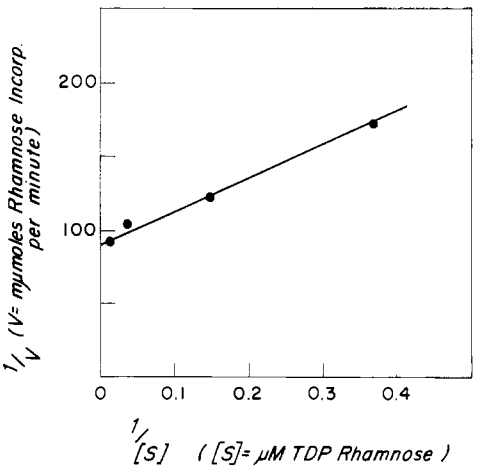


FIGURE 3: Relationship of the rate of incorporation to the concentration of substrate, TDPrhamnose. The reaction mixture contained, in a total volume of 0.20 ml, Tris-maleate buffer, *p*H 6.5, 10 μmoles; [$^{14}$C]-TDPrhamnose (specific activity 1.33 × $10^6$ cpm/μmole), as indicated; $MnCl_2$, 0.5 μmole; and "enzyme" from TV 208, 1.05 mg protein. Incubation was for 2 minutes at 0°.

[2] However, particulate fractions from LT2 did incorporate [$^{14}$C]L-rhamnose when the mixture of appropriate nucleotide-sugars was added. The details will be reported later (Y. Naide and H. Nikaido, to be published).

TABLE II: Strain Specificities of Rhamnose Incorporation Reaction.

| Strain | Specific Activity TDPG → TDP-rhamnose[a] (mμmoles/mg protein/hr) | Specific Activity Incorporation of Rhamnose[b] (μμmoles/mg protein) |
|---|---|---|
| *S. typhimurium* LT2 | 45 | <1 |
| *S. typhimurium* TV 160 | 47 | <1 |
| *S. typhimurium* TV 119 | 31 | <1 |
| *S. typhimurium* TV 208 | <1 | 291 |

[a] The enzymatic conversion of TDPG into TDP-rhamnose through TDP-4-keto-6-deoxyglucose. The enzyme(s) converting TDPG into the intermediate was always present in excess in these extracts. The results are from Nikaido *et al.* (1964). The enzymes used were the supernatants after centrifugation of crude sonic extracts at 12,000 × *g* for 10 minutes. [b] "Enzymes" used were the particulate fractions prepared as described in Methods. The incorporation experiments were done as described in Methods, with incubation at 37° for 15 minutes.

unit(s) more proximal to rhamnose (see also Discussion).

*Identification of the Reaction Product as LPS.* The washed particulate fraction (8 mg of protein) from TV 208 was incubated with [$^{14}$C]TDPrhamnose (7 mμmoles, 7 × $10^4$ cpm), $MnCl_2$ (0.5 μmole), and Tris-HCl buffer, *p*H 7.5 (30 μmoles), for 30 minutes at 37° in a total volume of 0.48 ml. The reaction was stopped with 5% trichloroacetic acid as described. The washed trichloroacetic acid precipitate was suspended in water and was neutralized with $Na_2CO_3$. An equal volume of 90% phenol was added, and the mixture was kept at 65–68° for 5 minutes. After cooling and centrifugation, the aqueous phase was removed, and the phenol phase was extracted twice more with water. The combined aqueous phase was dialyzed against water. The nondialyzable fraction thus obtained was called "crude LPS" here. Almost all of the radioactivity (81–97% in several experiments) of the trichloroacetic acid-precipitate fraction was extracted into the aqueous phase and was recovered in the "crude LPS" fraction, which in this experiment contained 3700 cpm. This behavior is characteristic of LPS (Westphal *et al.*, 1952a), as well as of RNA and other polysaccharides.

Four milligrams of purified, nonradioactive TV 208 LPS was added to an aliquot of the "crude LPS" as carrier, and the solution was subjected to cycles of ultracentrifugation each time at 115,000 × *g* for 2 hours. It is known that LPS, but not RNA or soluble polysaccharides, is sedimented under these conditions (Westphal *et al.*, 1952b), and it can be seen from Table III that most of the radioactivity was sedimented. Although a small proportion of radioactivity was lost to the supernatant at each centrifugation, the sedimentation of the carrier LPS was also incomplete, as is shown by the quantitative determination of hexose in LPS. The small discrepancy between the rates of loss of carrier LPS and of radioactive reaction product may be explained as follows. LPS forms polydisperse aggregates in water (Schramm *et al.*, 1952). "Carrier LPS" used in this experiment had been purified by repeated cycles of ultracentrifugation and may have consisted predominantly of larger aggregates compared with the "natural" LPS. Thus the carrier LPS was recovered more completely during this experiment than the polydisperse incorporation product, i.e., "natural" LPS.

LPS carries the immunological specificity of O or R antigen, and is therefore specifically precipitated with O or R antiserum. "Crude LPS" was mixed with 0.5 ml of a rabbit R antiserum prepared against an $R_{II}$-type rough mutant which is serologically very closely related to TV 208. The mixture was kept for 4 days at 0°, then

TABLE III: Ultracentrifugation of the Incorporation Product.[a]

| Step | Radioactivity cpm | Radioactivity Recovery[b] (%) | Carrier LPS[c] Glucose eq (μg) | Carrier LPS[c] Recovery[b] (%) | Specific Radioactivity (cpm/mg glucose eq) |
|---|---|---|---|---|---|
| Before ultracentrifugation | 1570 | | 818 | | 1920 |
| Sediment of first ultracentrifugation | 1110 | 71 | 745 | 91 | 1490 |
| Sediment of second ultracentrifugation | 897 | 81 | 550 | 74 | 1630 |
| Sediment of third ultracentrifugation | 842 | 94 | 520 | 94 | 1620 |

[a] "Crude LPS" isolated from the incubation mixture containing [$^{14}$C]TDPrhamnose was mixed with nonradioactive carrier LPS from TV 208 and was centrifuged at 115,000 × *g* for 2 hours in a SW-39 rotor of Spinco centrifuge. Total volume of samples centrifuged was 3.4 ml. The sediment was resuspended in distilled water and was centrifuged again. This operation was repeated three times. The values are corrected for loss due to the withdrawal of samples for analysis. For details see text. [b] Recovery from the previous step. [c] Assayed by anthrone method (Scott and Melvin, 1953) using D-glucose as standard.

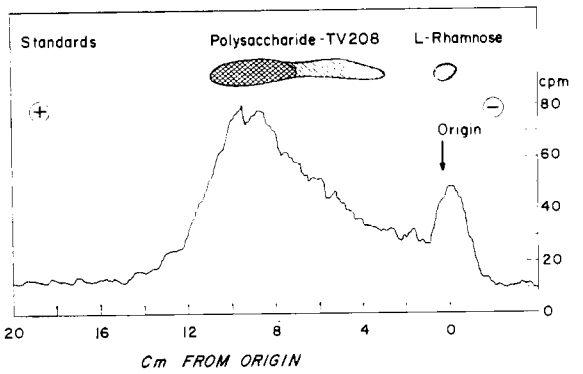


FIGURE 4: Paper electrophoresis of the products of incorporation. "Crude LPS" was isolated, as described in the text, from the incubation mixture containing [$^{14}$C]TDPrhamnose and "enzyme" from TV 208. It was treated with 1 N acetic acid at 100° for 30 minutes and, after the removal of water-insoluble residue, the sample was subjected to paper electrophoresis at *p*H 3.5 (buffer A) for 1 hour at the potential gradient of 70 v/cm.

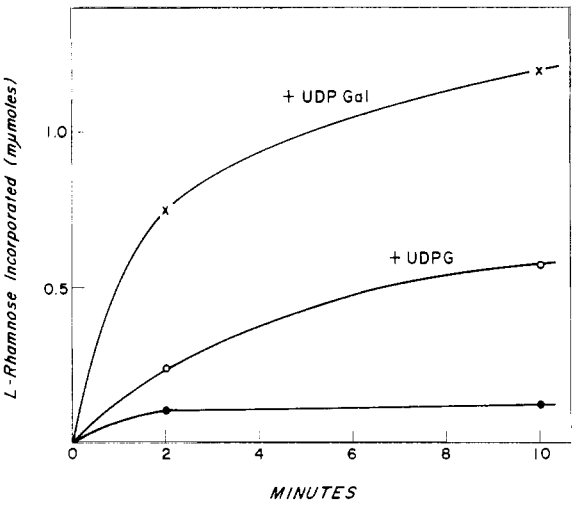


FIGURE 5: Effect of UDPGal and UDPG on the incorporation of [$^{14}$C]L-rhamnose from TDPrhamnose. Before use, "enzyme" from TV 208 was washed three times by resuspension and centrifugation in 0.05 M Tris-HCl buffer, *p*H 7.5. The incubation mixture contained, in 0.30 ml, Tris-maleate buffer, *p*H 6.6, 15 μmoles; $MnCl_2$, 1.0 μmole; [$^{14}$C]TDPrhamnose (specific radioactivity, 6.4 × $10^5$ cpm/μmole), 7 mμmoles; and enzyme, 2.1 mg protein. Amounts of UDPG and UDPGal added were 50 and 43 mμmoles, respectively.

centrifuged, the precipitate was washed three times with ice-cold 0.9% NaCl, and the final precipitate was plated and counted; 96% of the initial radioactivity was recovered. Similarly, 89% of the radioactivity was precipitated when the preparation purified by three successive ultracentrifugations was used instead of "crude LPS."

Another aliquot of the "crude LPS" from the enzymic incorporation experiment was heated at 100° for 30 min. in 1 N acetic acid, in order to dissociate LPS into lipid A and acidic polysaccharide (Westphal and Lüderitz, 1954). The water-soluble fraction containing polysaccharide was subjected to paper electrophoresis at *p*H 3.5. Most of the radioactivity moved toward the anode (Figure 4), and the mobility of the main peak was close to that of the polysaccharide obtained from the LPS of TV 208. The small amount of radioactivity remaining at the origin was shown to be free rhamnose released during the acid treatment. The radioactive compound(s) which moved toward the anode during electrophoresis did not move at all when eluted and chromatographed on paper; hence the size of these compounds is probably fairly large.

These findings indicate that [$^{14}$C]rhamnose was incorporated into the polysaccharide portion of the LPS. Furthermore, when the "crude LPS" was hydrolyzed with 1 N $H_2SO_4$ for 2 hours only one radioactive substance was released. It co-chromatographed with L-rhamnose in solvents A, B, and C, indicating that [$^{14}$C]-L-rhamnose was incorporated as such without further chemical alteration.

*Stimulation of* [$^{14}$C]L-*Rhamnose Incorporation by UDPGal.* As mentioned above, the addition of non-radioactive UDPGal or UDPG increased the saturation level of [$^{14}$C]rhamnose incorporation by the particulate fraction from TV 208. The degree of stimulation varied somewhat with the enzyme preparation used, and was in the range of five- to fifteenfold. In contrast, guanosine diphosphate mannose did not have any marked effect on the rhamnose incorporation system, nor did it stimulate the incorporation further in the presence of UDPGal or UDPG.

UDPG and UDPGal were effective to the same degree, but this was not unexpected since the particulate fraction of TV 208 was found to contain active UDPGal-4-epimerase catalyzing the interconversion of these two compounds, when assayed as described by Maxwell *et al.* (1962). That UDPGal is the active compound was suggested by the following observations. (1) When the particulate "enzyme" was extensively washed to remove as much UDPGal-4-epimerase as possible, and was then used in the incorporation experiment, UDPGal was much more active than UDPG, especially in the earlier phases of incubation (Figure 5). (2) A double mutant of TV 208 lacking UDPGal-4-epimerase, isolated by Stocker, was grown for 30 min. in the presence of galactose. In analogy with our previous results (Fukasawa and Nikaido, 1960; Nikaido, 1961), we expected that the resulting cells would have an LPS indistinguishable from that of TV 208. When the particulate fraction prepared from these cells (2.0 mg protein) was incubated for 15 min. at 37° in the reaction mixture described in the legend to Figure 5, the amounts of [$^{14}$C]rhamnose incorporated were as follows: with TDPrhamnose alone added as substrate, 0.02 mμmole; with the addition of UDPG, 0.03 mμmole; and with UDPGal, 0.38 mμmole. Thus, only UDPGal stimulated the incorporation in this system.

The [$^{14}$C]rhamnose incorporation product obtained in the presence of nonradioactive UDPGal was also subjected to the various treatments described above in Identification of the Reaction Product as LPS. It behaved in exactly the same way as the incorporation product obtained in the absence of UDPGal; it was extracted into the aqueous phase with 45% phenol, was nondialyzable, was sedimented by ultracentrifugation and with specific anti-$R_{II}$ serum, and gave rise to acidic polysaccharides after treatment with acetic acid.

The following experiment indicates that most of [$^{14}$C]-L-rhamnose incorporated under these conditions was present as a nonreducing terminal sugar.[3] [$^{14}$C]TDP-rhamnose was prepared from uniformly labeled [$^{14}$C]D-glucose. The particulate fraction of TV 208 (19.5 mg protein) was incubated for 30 min. at 37° with this substrate (67 mμmoles, $1.6 \times 10^4$ cpm), nonradioactive UDPGal (0.4 μmole), $MnCl_2$ (4 μmole), and Tris-HCl buffer, *p*H 7.5 (80 μmoles), in a total volume of 1.5 ml. "Crude LPS" was prepared from the trichloroacetic acid–insoluble fraction and was oxidized with periodate, and "formic acid" was isolated as described in Methods. The original, "crude LPS" fraction had radioactivity of 1960 cpm. From this, "formic acid" was isolated as volatile acid which counted at 356 cpm. Formic acid should be produced only from C-3 of nonreducing terminal rhamnose and not at all from nonterminal rhamnose;[4] hence if all of the rhamnose incorporated exists as nonreducing terminal units, $1960 \times {}^1/_6 = 327$ cpm should be released as formic acid, assuming uniform distribution of label in the [$^{14}$C]glucose used. The value obtained was thus 109% of the theoretical.[5]

*Incorporation of* [$^{14}$C]D-*Galactose.* When the particulate fraction from TV 208 was incubated with [$^{14}$C]UDP-Gal, radioactive material was incorporated into the trichloroacetic acid–insoluble fraction. Furthermore, as UDPGal enhanced the incorporation of [$^{14}$C]L-rhamnose, nonradioactive TDPrhamnose also enhanced the incorporation of "[$^{14}$C]galactose" from [$^{14}$C]UDP-Gal (Table IV). The amount of "galactose" incorporated was of the same order of magnitude as the amount of rhamnose incorporated at the same time. When "crude LPS" was isolated after the incubation of particulate enzyme with [$^{14}$C]UDPGal, and was hydrolyzed with acid, radioactivity was found to be present both in glucose and galactose moieties of LPS. This is due to the conversion of [$^{14}$C]UDPGal into [$^{14}$C]UDPG by UDPGal-4-epimerase during incubation, but it is noteworthy that only the incorporation of [$^{14}$C]galactose was stimulated by the addition of nonradioactive TDPrhamnose (Table V). From the relationship with [$^{14}$C]rhamnose incorporation, it seems probable that galactose is also incorporated into LPS, while [$^{14}$C]glucose might be incorporated into another polymer.

TABLE IV: Interdependence of [$^{14}$C]Rhamnose and "[$^{14}$C]Galactose" Incorporation.[a]

| Nucleotide-Sugar Added | | Incorporation into Trichloroacetic Acid–insoluble Fraction (mμmoles) | |
|---|---|---|---|
| $^{14}$C | $^{12}$C | Expt 1 | Expt 2 |
| TDPrhamnose | None | 0.4 | 0.7 |
| TDPrhamnose | UDPGal | 3.1 | 3.0 |
| UDPGal | None | 2.3 | 0.9 |
| UDPGal | TDPrhamnose | 3.3 | 2.9 |

[a] The reaction mixture contained Tris-HCl buffer, *p*H 7.5, 15 μmoles; $MnCl_2$, 0.5 μmole; washed "enzyme" from TV 208 (4.0 mg protein in expt. 1, 4.5 mg protein in expt 2). The amounts of nucleotides were: UDPGal, 35 mμmoles; TDPrhamnose, 10.5 mμmoles. The specific radioactivity of nucleotides was: UDPGal, $2.3 \times 10^5$ cpm/μmole; TDPrhamnose, $6.4 \times 10^5$ cpm/μmole. The incubation was at 37° for 15 minutes.

*Partial Acid Hydrolysis of Incorporation Product.* The washed particulate fraction from TV 208 (8.8 mg protein/tube) was added to two tubes each containing Tris-HCl buffer, *p*H 7.5 (30 μmoles), $MgCl_2$ (1 μmole), and [$^{14}$C]UDPG-Gal (44 mμmoles, $3.8 \times 10^5$ cpm). To one tube (A), [$^{12}$C]TDPrhamnose (10 mμmoles) was also added; to another (B), [$^{14}$C]TDPrhamnose (10 mμmoles, $10^5$ cpm). After 30 minutes incubation at 37°, the reaction was stopped by the addition of 5% trichloroacetic acid. "Crude LPS" preparations were isolated as described in Identification of the Reaction Product as LPS. "Crude LPS A" (36,500 cpm) and "B" (86,800 cpm) were partially hydrolyzed by heating with 1 N $H_2SO_4$ for 16 min. at 100°. The acid was removed by treatment with Dowex 1 ($HCO_3^-$), and the supernatant was chromatographed on Whatman 3MM paper with solvent A. Besides the radioactivity remaining at the origin, the only radioactive band found had the same $R_F$ as D-galactose (in the case of partial hydrolysate from "crude LPS B," a band of [$^{14}$C]rhamnose was also seen). The "galactose" band was eluted with water.

[3] This conclusion was also confirmed by the partial acid hydrolysis of incorporation products. If rhamnose is incorporated as a subterminal sugar, partial acid hydrolysis of the incorporation product should yield oligosaccharides of glycosyl rhamnose type, because rhamnosyl linkages are acid labile. However, oligosaccharides of this type were not found (see Partial Acid Hydrolysis of Incorporation Product).

[4] This assumption is valid only if no overoxidation had occurred. The following results suggest that overoxidation is unlikely under these conditions. (1) When the purified LPS from TV 208 was oxidized under the same conditions and the time course of periodate consumption was followed, we found that only 1.4 moles of periodate was taken up per mole monosaccharide in LPS, and that little further consumption took place after 3 days. (2) At 24 hours, the consumption of periodate reached 85% of the final value. When the oxidation was stopped at this time in the experiments with radioactive "crude LPS," we found that 72% of the theoretical amount of "[$^{14}$C]formic acid" had already been released.

[5] The manufacturer of the [$^{14}$C]D-glucose states that 15% is the maximum deviation of labeling from uniformity.

TABLE V: Effect of TDPrhamnose on the Incorporation of [$^{14}$C]Glucose and [$^{14}$C]Galactose in the Presence of [$^{14}$C]UDPGal.[a]

| Additions to the Reaction Mixture | Incorporated $^{14}$C Recovered as Glucose (mμmole) | Galactose (mμmole) |
|---|---|---|
| None | 0.43 | 0.19 |
| [$^{12}$C]TDPrhamnose | 0.52 | 0.66 |

[a] The reaction mixture contained Tris-maleate buffer, *p*H 6.6, 25 μmoles; $MnCl_2$, 0.5 μmole; [$^{14}$C]UDPGal, 0.14 μmole (specific radioactivity, 2.3 × $10^5$ cpm/μmole); "enzyme" from TV 208, 12.8 mg protein; in a final volume of 0.5 ml. TDPrhamnose, 14 mμmoles, was added to one of the tubes. After incubating for 15 minutes at 37°, "crude LPS" was isolated as described in Methods, and contaminating nucleic acids were removed by incubating with DNAase and RNAase. Following dialysis the samples were hydrolyzed with 1 N $H_2SO_4$ for 4 hours at 100°. After removal of the acid, the hydrolysates were electrophoresed on Whatman 3MM paper for 3 hours at 36 v/cm using buffer D. Glucose and galactose formed well-separated bands, and these bands were cut out and radioactivity was determined with a Tri-Carb liquid scintillation counter.

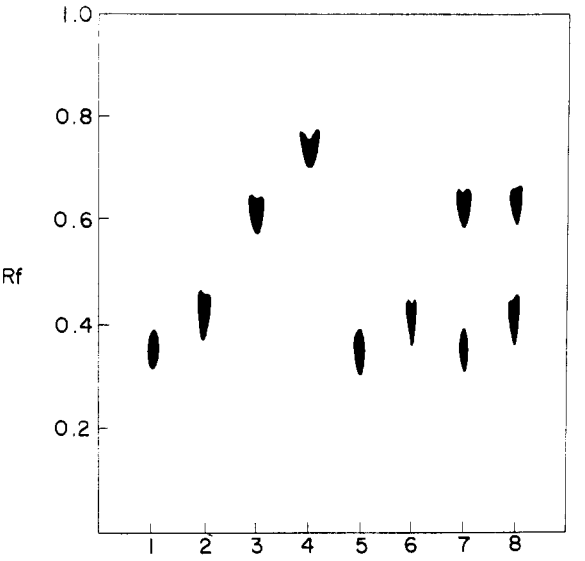


FIGURE 6: Thin-layer chromatography of the hydrolysates of X and reduced X. Samples were prepared as described in text. Before chromatography, hydrolysates of reduced X were further treated with Dowex 50 ($H^+$) and then evaporated with methanol to remove borate. 1, D-galactose; 2, galactitol; 3, L-rhamnose; 4, L-rhamnitol; 5, hydrolysate of X from "crude LPS A"; 6, hydrolysate of reduced X from LPS "A"; 7, hydrolysate of X from LPS "B"; 8, hydrolysate of reduced X from LPS "B". Standards (1–4) were revealed by staining with $AgNO_3$ (Trevelyan *et al.*, 1950). Radioactive material in hydrolysates (5–8) was detected with autoradiography.

and the eluate was rechromatographed on Whatman 3MM paper strips with solvent D for 48 hours at room temperature. Two radioactive bands were observed. One had the same $R_F$ as D-galactose and migrated 25 cm from the origin. The other, called X, migrated 19.5 cm from the origin. Total radioactivity of X obtained from "crude LPS A" was 550 cpm; that from "crude LPS B," 1860 cpm. An aliquot of X eluted from the paper strip was rechromatographed in solvent B, and another aliquot was subjected to paper electrophoresis with buffer B for 90 min. at the potential gradient of 60 v/cm. In both cases X behaved as a single substance, and no contamination was found; in electrophoresis, X moved 7 cm from the origin toward the anode, while galactose (used as a control) moved 13.5 cm. Aliquots of X were reduced with $NaBH_4$ (10 mg) overnight at 4°. Aliquots of both X and reduced X were hydrolyzed with 1 N HCl for 4 hours at 100°, and HCl was removed by repeated evaporation to dryness *in vacuo*. The samples were then chromatographed on thin layers of cellulose using solvent B, and radioactive spots were detected by autoradiography with Kodak no-screen X-ray film.

The results, shown in Figure 6, indicate clearly that X contains both nonreducing rhamnose and reducing galactose moieties. In addition, radioactive substances in the hydrolysate of X (from "crude LPS B") co-chromatographed with D-galactose and L-rhamnose in solvents A, B, and D. Since the difference in $R_F$ between D-galactose and galactitol is rather small in solvent B, the samples were further examined by electrophoresis. Hydrolysates of both X and reduced X from "crude LPS A" were subjected to paper electrophoresis. The results, shown in Figure 7, confirm the conclusion that most of the [$^{14}$C]galactose in X exists as reducing terminal units. When the hydrolysate of X from "crude LPS B" was chromatographed on paper with solvent A, and the [$^{14}$C]rhamnose and [$^{14}$C]galactose spots were cut out and counted, the former had 210 cpm and the latter 183 cpm. Assuming that their specific radioactivity is the same as that of [$^{14}$C]TDPrhamnose and [$^{14}$C]UDPG-Gal used in the incubation, the molar ratio of rhamnose to galactose in X is 1.00:1.02.

These results strongly suggest that X is a disaccharide, rhamnosylgalactose. The possibility that another nonradioactive sugar might be present in X appears rather unlikely in view of the high $R_F$ of X in solvents A, B, and D.

## Discussion

Studies have been made, in this as well as in other laboratories, on various mutants of *S. enteritidis* and *S. typhimurium* blocked in the biosynthesis of cell wall LPS. Figure 8 shows a hypothetical structure of LPS in

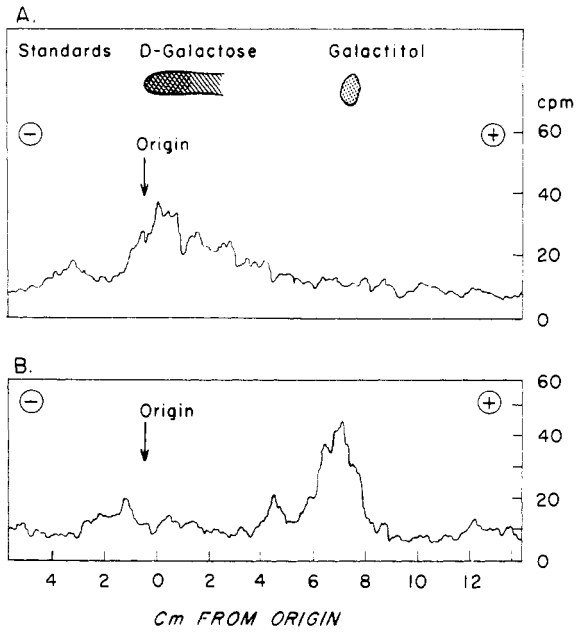


FIGURE 7: Paper electrophoresis of hydrolysates of X and reduced X. Compound X containing [$^{14}$C]-galactose was isolated from "crude LPS A", and aliquots, before and after reduction with $NaBH_4$, were hydrolyzed with 1 N HCl for 4 hours. After removal of HCl *in vacuo* followed by treatment with Dowex 50 ($H^+$) and evaporation with methanol to remove sodium borate, the samples were electrophoresed with buffer C for 90 minutes at the potential gradient of 25 v/cm. Paper strips were scanned with a Vanguard 4 $\pi$ scanner. Standards were located by staining with $AgNO_3$ (Trevelyan *et al.*, 1950). For details see text.

these bacteria, based on these studies (for review, see Osborn *et al.*, 1964) and the results reported here.

When a mutant defective in phosphoglucoisomerase, which cannot make UDPG from carbon sources other than glucose, is grown in the absence of glucose, the synthesis of LPS stops before the addition of glucose, leaving an incomplete LPS containing only heptose, phosphate, and 2-keto-3-deoxyoctonate in its polysaccharide portion (Fraenkel *et al.*, 1963). Recently, an additional component, *O*-phosphorylethanolamine (Grollman and Osborn, 1964), was found to be present in this LPS.

Mutants defective in UDPGal-4-epimerase cannot make UDPGal, and they synthesize LPS containing glucose in addition to heptose, phosphate, and 2-keto-3-deoxyoctonate (Nikaido, 1962a; Osborn, 1963). A glucosylheptose was isolated from a partial acid hydrolysate of such LPS (H. Nikaido, unpublished), and it seems therefore certain that glucose ($Glu_1$ of Figure 8) is attached to heptose.[6] Particulate fractions of these mutants catalyze the transfer of galactose from UDPGal onto the incomplete LPS (Nikaido, 1962b; Osborn *et al.*, 1962). It was further shown that another glucose residue ($Glu_2$ of Figure 8), followed by *N*-acetylglucosamine, can be added onto this galactose by the same enzyme preparation (Osborn *et al.*, 1964). A similar finding was reported on a mutant of *E. coli* (Edstrom and Heath, 1964).

It is known that rough mutants of *Salmonella* can be divided into two groups, $R_I$ and $R_{II}$, by virtue of the different serological specificity of their LPS (Beckmann *et al.*, 1964b). It was also established that rough mutants whose genetic mutation is located in the *rouA* region produce $R_I$ LPS, and in the *rouB* region, $R_{II}$ LPS (Beckmann *et al.*, 1964a). The reason for assuming that *rouA* ($R_I$) should be blocked deeper than *rouB* ($R_{II}$) has been already stated (Nikaido *et al.*, 1964). It should be added that Lüderitz *et al.* (1964) have recently arrived at the same conclusion through the study of *Salmonella minnesota* rough mutants.

Strain TV 208 cannot make TDPrhamnose and synthesizes an incomplete, $R_{II}$-type LPS. If the monosaccharide units are added in a completely sequential manner, as was the case with $Glu_I$, $Gal_c$, $Glu_2$, and GNAc of Figure 8, then the first sugar attached to the $R_{II}$ core must be rhamnose. Although this hypothesis seemed initially to have been supported by the finding of the *in vitro* incorporation of [$^{14}$C]rhamnose with the particulate fractions of TV 208, it was subsequently shown to be incorrect. The first evidence which suggested this was the following. When TDPrhamnose was the only substrate added, the maximal amount of incorporation of [$^{14}$C]rhamnose was quite small compared with the other *in vitro* systems. In the galactosyl transfer system of epimerase-less mutants, about one galactose residue can be incorporated per five terminal glucose units of LPS contained in the enzyme preparation (H. Nikaido, unpublished). In the TV 208 system, only one rhamnose residue was incorporated per 200–1000 heptose units of endogenous $R_{II}$ LPS. Since it is known that there exists one terminal *N*-acetylglucosamine unit/two heptose units in $R_{II}$ LPS (Osborn *et al.*, 1964), this means the incorporation of one rhamnose residue/100–500 terminal units. Furthermore, we have shown in this paper that UDPGal greatly increased the amount of [$^{14}$C]rhamnose incorporation, and under such conditions [$^{14}$C]galactose was also incorporated into LPS in amounts approximately equimolar to rhamnose; yet all the rhamnose was incorporated as nonreducing terminal units.

We can explain these data by assuming that (1) galac-

[6] In a previous communication (Nikaido, 1962a), results from another laboratory were quoted which indicated the presence of $\beta$-1,2- and $\beta$-1,6-glucosylglucose linkages in the LPS from an epimeraseless mutant. However, the method used for the isolation of "LPS" makes one suspect that what was believed to be LPS was actually some other soluble glucan. From hydrolysates of LPS from an epimeraseless *S. enteritidis*, we have isolated $\alpha$-1,4-glucosylglucose (maltose), but not $\beta$-1,2-glucosylglucose (sophorose) or $\beta$-1,6-glucosylglucose (gentiobiose). The localization of this glucosylglucose linkage in the over-all scheme of LPS structure (Figure 8) is obscure; it is quite possible that the maltose came from contamination of the LPS preparation with glycogen.

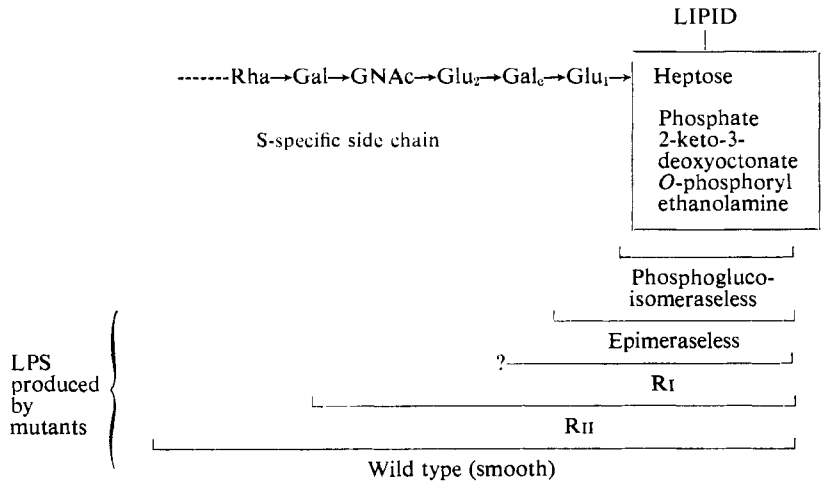


FIGURE 8: Hypothetical structure of LPS from various mutants of *S. typhimurium*. This scheme is based on the studies reported here as well as studies from our own and other laboratories, for which references are given in the text. Abbreviations: Rha, L-rhamnose; Gal, D-galactose; GNAc, *N*-acetyl-D-glucosamine; Glu, D-glucose.

tose is the first sugar to be attached to $R_{II}$ LPS, (2) rhamnose is attached onto this galactose unit, and (3) the addition of the galactose unit proceeds very poorly in the absence of TDPrhamnose. According to this hypothesis, very few galactose units would be attached to the $R_{II}$ LPS in TV 208 cells because TDPrhamnose is absent. Therefore, the LPS of TV 208 remains predominantly of $R_{II}$ type in its serological and chemical properties. The sequence of transfer proposed above is also strongly supported by the isolation of rhamnosylgalactose from partial hydrolysates of the incorporation product.[7]

At present, however, we do not have any evidence to explain why the presence of TDPrhamnose stimulates the transfer of galactose. Two hypotheses can be proposed. First, it could be that these sugar units become linked on an intermediate carrier to form a carrier oligosaccharide complex, and that the oligosaccharide is transferred as such to LPS. Loss of any component sugar in the oligosaccharide would then impair the synthesis and subsequent transfer of the whole oligosaccharide. According to this hypothesis, the carrier molecules in TV 208 cells would be fully loaded with galactose, because galactose alone presumably could not be transferred from the carrier to the $R_{II}$ LPS. When only [$^{14}$C]TDPrhamnose is added to the particulate fraction of this strain, [$^{14}$C]rhamnose would be added to the galactose on the carrier, and the rhamnosylgalactose disaccharide would then be transferred to the $R_{II}$ LPS. Thus the incorporation of [$^{14}$C]rhamnose would stop when the small supply of galactose carrier complex is used up. In contrast, when UDPGal is added together with [$^{14}$C]TDPrhamnose, the galactose carrier complex would be constantly regenerated by the transfer of galactose from UDPGal to the free carrier, and the incorporation of rhamnose into LPS would continue much longer.

Alternatively, it can be assumed that these sugars are transferred to LPS sequentially as monosaccharides, and that TDPrhamnose behaves as an activator of the enzyme catalyzing the transfer of galactose onto $R_{II}$ LPS. According to this hypothesis, only a few galactosyl units would be transferred to the $R_{II}$ LPS in TV 208 cells, because of the absence of the activator. When [$^{14}$C]TDPrhamnose alone is added to the particulate fraction, rhamnose would be transferred onto these few galactosyl units on the LPS, and the available acceptor sites would be saturated very quickly. When both UDPGal and TDPrhamnose are added, galactose would be transferred first to $R_{II}$ LPS, thus creating a large number of attachment sites for rhamnose, and rhamnose would then be added on.[8] Further study is necessary to decide between these hypotheses.

Although a rhamnosylgalactose unit was found to be

[7] The small yield of rhamnosylgalactose after partial acid hydrolysis was not unexpected, because rhamnosyl linkages are generally known to be acid labile. Nevertheless, in view of this small yield, the isolation of the disaccharide should not be taken as an unequivocal proof that *all* the rhamnose units were incorporated in the proposed sequence. For instance, we cannot exclude the possibility that some of the rhamnose units are attached directly to the $R_{II}$ LPS without the intervention of galactose.

[8] When the particulate fraction was incubated first with UDPGal alone, washed, and then incubated with [$^{14}$C]TDP-rhamnose alone, the preparation incorporated no more $^{14}$C than did the control which had been incubated without UDPGal in the first step. This finding was predicted by our hypothesis, since in the absence of TDPrhamnose very little galactose should be incorporated into $R_{II}$-type LPS. Although some enzyme preparations incorporate fair amounts of radioactivity from [$^{14}$C]UDPGal in the absence of TDPrhamnose, most of the radioactivity is frequently incorporated as [$^{14}$C]glucose (see Table V, for example) perhaps into a non-LPS polymer. A portion of the [$^{14}$C]galactose might also be incorporated into such a polymer unrelated to LPS.

incorporated, we could not establish the exact site of its attachment to $R_{II}$-type LPS. The following considerations, however, suggested to us that most probably the galactose is linked to *N*-acetylglucosamine terminal units as shown in Figure 8. (1) These *N*-acetylglucosamine units appear to be the determinant group of $R_{II}$ immunological specificity (Lüderitz *et al.*, 1965). (2) They seem to be *completely covered* in S-type LPS, because S-type LPS of *S. typhimurium* does not react with anti-$R_{II}$ serum (Y. Naide and H. Nikaido, unpublished; also see Lüderitz *et al.*, 1964). Probably the S-specific side chains are attached onto these *N*-acetylglucosamine units. (3) It was shown (Nikaido and Nikaido, 1965) that abequose and mannose are further transferred onto rhamnosylgalactosyl-($R_{II}$ LPS) *in vitro*, exactly in the sequence comprising the repeating unit of the S-specific side chain. Thus, the rhamnosylgalactose unit appears to constitute the innermost portion of the side chain and, accordingly, should be linked to the *N*-acetylglucosamine unit. It should be emphasized, however, that this argument involves several unverified assumptions. For example, *N*-acetylglucosamine units in S-type LPS might still be unsubstituted but inaccessible for the antibody because of steric hindrance. The elucidation of these points must await further chemical studies, which are in progress.

It should be mentioned that TDPrhamnose can act as the rhamnosyl donor in the synthesis of rutin (Barber, 1962), rhamnolipid (Burger *et al.*, 1962), and streptococcal cell wall polysaccharide (Pazur and Anderson, 1963). The transfer of L-rhamnose from TDPrhamnose into LPS was also reported recently using the particulate fraction of a mutant of *S. typhimurium* deficient in guanosine diphosphate mannose (Osborn *et al.*, 1964).

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# Synthesis of Trinucleoside Diphosphates with Polynucleotide Phosphorylase*

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ABSTRACT: Highly purified, primer-requiring preparations of polynucleotide phosphorylase have been used to synthesize triribonucleoside diphosphates of specific base composition and sequence. In this procedure, a particular nucleoside 5′-diphosphate is treated with a dinucleoside monophosphate under conditions favoring the synthesis of trinucleoside diphosphate rather than long-chain polymers.

The synthesis and characterization of fourteen of the sixty-four possible common trinucleoside diphosphates are reported. The method appears to be completely versatile.

In this paper we would like to report a general enzymic method for the synthesis of trinucleoside diphosphates of known sequence. The procedure is based on earlier work (Singer *et al.*, 1960a,b) which demonstrated that polynucleotide phosphorylase can add one or more nucleotide residues (from a nucleoside 5′-diphosphate) onto a suitable preformed oligonucleotide chain. Oligonucleotides such as pApApApU[1] and pApApApGpG were prepared and characterized.

The reaction we have used can be described as follows:

$$\mathrm{XpY} + \mathrm{NDP} \xrightarrow[\text{phosphorylase}]{\text{polynucleotide}} \mathrm{XpYpN} + \mathrm{P_i} \quad (1)$$

In equation (1), XpY is a dinucleoside monophosphate, NDP a nucleoside 5′-diphosphate, and XpYpN the trinucleoside diphosphate product. The procedure is general in that any given trinucleoside diphosphate can be synthesized; however, certain specific conditions are necessary in particular cases. The success of reaction (1) in synthesizing reasonable yields of specific trinucleoside diphosphates depends on several factors. (1) Highly purified polynucleotide phosphorylase from *Micrococcus lysodeikticus* (Singer and Guss, 1962; Singer and O'Brien, 1963) has an absolute requirement for an oligonucleotide primer for the synthesis of polyribonucleotides under the conditions used. (2) Dinucleoside monophosphates, as well as dinucleotides, can serve as primers for the phosphorylase (Singer *et al.*, 1960a; Thach *et al.*, 1964). (3) Dinucleoside monophosphates are not subject to phosphorolytic cleavage by the phosphorylase (Singer, 1958). Therefore rearrangement of the nucleotide sequence of the primer either by breakdown and resynthesis or by transnucleotidation (Singer *et al.*, 1959) is not a problem. (4) Conditions which limit long-chain polymer synthesis and thereby permit the accumulation of short-chain products are required. Such conditions have been studied further and are reported in this paper.

The need for a method such as described in this paper arose from recent work on the genetic code. A rapid, sensitive method for measuring the m-RNA–

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[1] Abbreviations: A, U, G, and C represent the nucleoside residues adenosine, uridine, guanosine, and cytidine, respectively. For polyribonucleotides of specific structure the letter p to the left of the nucleoside initial indicates a 5′-phosphate, the letter p to the right, a 3′-phosphate (see Heppel *et al.*, 1957). Thus, ApG is adenylyl-(3′,5′)-guanosine, and pApG is 5′-*O*-phosphoryladenylyl-(3′,5′)-guanosine. An $A_{260}$ unit is the amount of material giving an absorbance of 1.0 in 1.0 ml of solution in a 1-cm light path. NDP represents a nucleoside 5′-diphosphate; ADP, UDP, GDP, CDP are the 5′-diphosphates of adenosine, uridine, guanosine, and cytidine, respectively; AMP, CMP, UMP, GMP are the 5′-phosphates of adenosine, cytidine, uridine, and guanosine, respectively.