THE DIRECTION OF CHAIN GROWTH IN *SALMONELLA ANATUM* O-ANTIGEN BIOSYNTHESIS

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The surface O-antigen of *S. anatum* is a polysaccharide with a linear mannosyl-rhamnosyl-galactosyl repeating sequence. It is synthesized from the sugar nucleotides GDPM†, TDPRh and UDPGal by prior formation of a trisaccharide which is linked through a pyrophosphate bridge to a polyisoprene alcohol (ACL) (Wright *et al.*, 1965a, 1967). Polymerization then occurs by the assembly of trisaccharide intermediates according to the reaction:

$$\text{Man-Rha-Gal-P-P-ACL} + (\text{Man-Rha-Gal})_n\text{-P-P-ACL} \longrightarrow (\text{Man-Rha-Gal})_{n+1}\text{-P-P-ACL} + \text{P-P-ACL}$$

There are two ways in which this reaction can occur. Either, (A), the trisaccharide can be split from its ACL pyrophosphate and added to the terminal mannose of the growing chain or, (B), the galactose end of the nascent chain can be transferred to the mannose of the trisaccharide. Experiments are reported here which show that the chains grow at their galactose ends and that they are, therefore, synthesized by mechanism B.

The two mechanisms were distinguished by pulse labeling antigen chains being synthesized *in vivo* or *in vitro* and, after isolation of the chains produced, examining the radioactivity

† Abbreviations used: ACL, antigen carrier lipid; Gal, D-galactose; GDPM, guanosine diphosphate-D-mannose; Man, D-mannose; Rha, L-rhamnose; TDPRh, thymidine diphosphate-L-rhamnose; UDPG, uridine diphosphate-D-glucose; UDPGal, uridine diphosphate-D-galactose.

incorporated into the terminal galactose. Parallel experiments in which the pulse of radioactivity was followed by a longer chase of non-radioactive precursor served as controls. By mechanism A above, the pulse should give little radioactivity in the terminal galactose in comparison to the other galactose residues of the chain, and this level should not be changed following the chase. By mechanism B, the pulse should radioactively label the terminal galactose preferentially, but following the chase this level should significantly fall.

In both types of experiment the strain used was L14, a rough mutant of S. anatum which is blocked in the transfer of completed O-antigen chains to the lipopolysaccharide core (Keller, 1966). Good yields of free antigen chains could thus be obtained

In vivo experiment

Glucose-fructose-^{14}C (0.2 ml; about 0.8 μmole and 2.4 x 10^8 cts/min) prepared as described previously (Wright and Robbins, 1965b), was added to each of 3 ,100 ml cultures of L14 (about 3 x 10^8 cells/ml) in L. broth. (Robbins et al., 1964). After 60 seconds one culture (pulse) was poured directly onto crushed ice, while to the other two (chase) non-radioactive glucose and fructose (560 μmoles of each) were added and growth continued for 5 minutes in one and 15 minutes in the other. After harvesting by centrifugation and washing with cold ethanol, the final cell pellet was suspended in 10 ml of cold water and extracted at 65°C with an equal volume of phenol (Westphal et al., 1952). This treatment, besides extracting the bulk of cellular protein into the phenol layer, will also split the labile linkage between the antigen chains and ACL. The resulting aqueous fractions were digested with E. coli alkaline phosphatase (Worthington Co.) to yield free antigen chains after extensive dialysis.

The ^{14}C-labeled preparations, each containing 1-2 x 10^6 cts/min, were reduced by exposure to 20 mc of tritiated $NaBH_4$ (New England Nuclear Co.) for 2 hours at room temperature. After reaction the samples were dialysed against 2% glycerol to remove

borate, and later against water. Charged components were then removed by passage through 1 x 7 cm. columns of DEAE (acetate form) and the samples applied to G75 sephadex columns with the results shown in Fig. 1a. All of the radioactivity, with the exception of a small ^{3}H peak at the included volume, was in high molecular weight material. Comparison of the $^{14}C/^{3}H$ ratios from two of the columns (Fig. 1b) shows that displacement of the ^{14}C activity to higher molecular weight species occurred during the

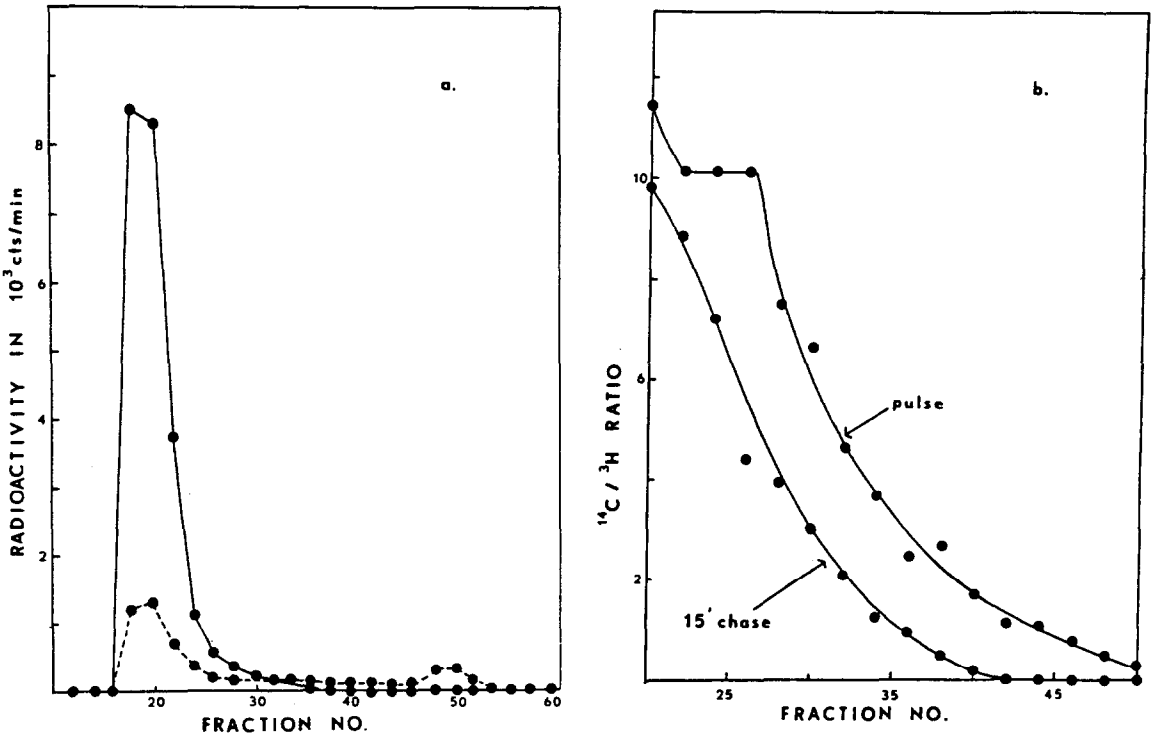

Figure 1a. ^{14}C and ^{3}H profiles of O-antigen chains fractionated on a 1 x 50 cm G75 Sephadex column. 1.0 ml fractions were collected. The profile shown is that of the 15 minute chase sample; the other two samples gave similar patterns.

——— ^{14}C ---------- ^{3}H

Figure 1b. The $^{14}C/^{3}H$ ratios from the above fractionations of the pulse sample and the 15 minute chase sample. The actual ratios in the chase have been multiplied by 1.41 to correct for the extra growth in this sample compared to the pulse (see text).

chase. The $^{14}C/^{3}H$ ratios are proportional to the ^{14}C activity per mole and are thus independent of individual losses in isolation. However, since the chase samples underwent 5 and 15 minutes longer growth, they had a larger amount of antigen unlabeled with ^{14}C but subsequently labeled with ^{3}H. The ratios in Fig. 1b and Table 1 have been normalized to the pulse sample assuming a generation time of 30 minutes.

The fractions containing the main peak of radioactivity were pooled for each of the samples and hydrolysed in 0.2N H_2SO_4 (overnight at 110°C). Following chromatography in solvent 1, n-butanol, pyridine, water (6:4:3), three radioactive peaks were detected corresponding in mobility to mannose, galactose (and galactitol) and rhamnose. Galactitol was separated from galactose by subsequent chromatography in solvent 2, isopropanol, water (4:1) and solvent 3, n-butanol-pyridine-0.05M morpholinium tetraborate, pH 8.6, and finally the ratio of $^{14}C/^{3}H$ in this compound determined. The results in Table 1 show that only one sample - that of the pulse - had an appreciable amount of ^{14}C activity and this is emphasized in the $^{14}C/^{3}H$ ratios. This is the result predicted for mechanism B above.

PERIOD OF CHASE	cts/min in galactitol ^{14}C	^{3}H	$^{14}C/^{3}H$ ratios observed	corrected
0	830	630	1.32	1.32 (1.0)
5 min	29	692	0.042	0.047 (1.1)
15 min	38	1,332	0.028	0.040 (1.4)

Table 1. Radioactivity of the galactitol from the *in vivo* experiment. The correction applied to the $^{14}C/^{3}H$ ratios is described in the text; the factors used are in parentheses.

In vitro experiment

It has been previously shown that *S. anatum* cells, frozen and thawed in 0.01M EDTA and incubated with TDPRh, GDPM and UDPGal, efficiently synthesize normal O-antigen (Robbins *et al.*, 1964). In order to repeat the above experiment *in vitro*, EDTA-

treated cells were prepared from L14 and incubated with ^{14}C-UDPGal as the radioactive precursor. Two incubation mixtures were prepared containing in 20 ml; 1.5 μmoles of TDPRh, 5 μmoles of GDPM, 2.5 mmoles of Tris-HCl pH 7.8, 1.0 mmole of $MgCl_2$ and about 40 mg of enzyme protein. After warming to 37°C, 0.3 ml of ^{14}C-UDPG-Gal† (about 96 mμmoles and 9.3×10^6 cts/min), prepared as described by Wright and Robbins (1965), were added to one mixture (pulse). Two minutes later, 10 ml of ice-cold 2N acetic acid were added to stop the reaction. The second mixture (chase) was incubated with ^{14}C-UDPG-Gal in the same way, but after 2 minutes non-radioactive UDPG-Gal (10 μmoles total) was added and the incubation continued for 20 minutes.

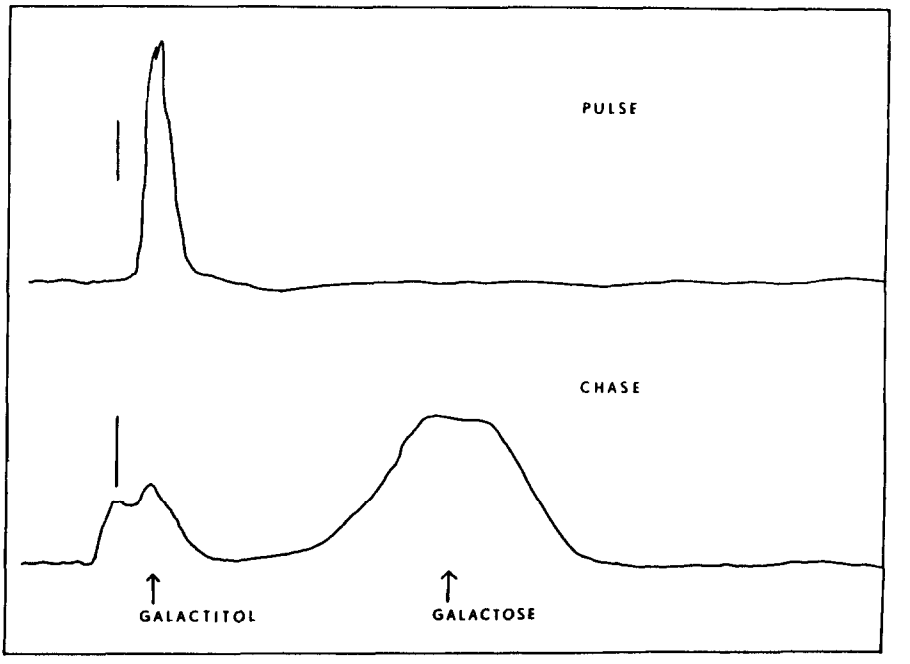

Figure 2. Radioactive profiles obtained from the in vitro experiment. Chromatography was in solvent 3.

† UDPG-Gal is the equilibrium mixture of UDPG and UDPGal produced by incubating UDPG with yeast UDP-galactose-4-epimerase. Only UDPGal is incorporated under the conditions used.

After centrifugation the pellets were suspended in water and extracted with hot phenol as before. Subsequent steps were the same as in the *in vivo* experiment except that reduction was carried out with non-radioactive $NaBH_4$ and the Sephadex fractionation step was omitted. The final preparation was hydrolysed in H_2SO_4 and chromatographed directly in solvent 3. The results in Fig. 2 show that from the pulse sample only galactitol was recovered while from the chase the major component was galactose, with only a minor amount of galactitol. This clearly indicates that *in vitro* as *in vivo* the radioactivity proceeds first into the galactose end of the polymer.

From these results it seems reasonable to suppose that chain growth in *S. anatum* occurs at the galactose or 'reducing' end. Such a mechanism is novel to the biosynthesis of polysaccharides in which it is often assumed that growth occurs from the 'non-reducing' end as in glycogen. On the other hand the method of chain elongation of proteins and fatty acids is directly comparable. It will be interesting to see how many other polysaccharides are synthesized in this way. (Robbins *et al.*, 1967).

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