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SYNTHESIS WITH IMMOBILIZED ENZYMES OF TWO TRISACCHARIDES, ONE OF THEM ACTIVE AS THE DETERMINANT OF A STAGE ANTIGEN.

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Summary. Glycosidation of disaccharides I and III with a system of five immobilized enzymes gave excellent yields of trisaccharides β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)-D-Gal |the I(Ma) determinant| and β-D-Gal-(1→4)-β-D-Glc-NAc-(1→3)-D-Gal, on the milimole scale.

According to H. Paulsen,[1] "... each oligosaccharide synthesis remains an independant problem whose resolution requires considerable systematic research and a good deal of knowhow". Thus the scaling up of enzymatic methods and the purification of more glycosyltransferases appear to be valuable objectives. In any case, the domestication of enzymes by the organic chemist is a matter of current interest. β-D-Galactosyltransferase catalyses the transfer of a β-D-galactopyranosyl unit from UDP-galactose[2] to position 4 of N-acetylglucosamine. The precursor, UDP-glucose (in the presence of epimerase) was obtained by two of us in 1982,[3] in 100% yield on the milimole scale, from glucose α-1-phosphate and UDP, with acetyl phosphate as a source of energy and three immobilized enzymes. Meanwhile, the group of G.M. Whitesides reported[4] an efficient synthesis of N-acetyllactosamine, with regeneration of cofactors, using five enzymes immobilized on a polyacrylamide gel,[5] and the more convenient phospho-enolpyruvate as a source of energy. In this letter we describe the preparation of trisaccharides II and IV (Figures 1 and 2) by operation of a similar system on the chemically-synthesized disaccharides I and III. Because of its local availability, a very different insoluble support was utilized, the polysaccharide Ultrogel.

Trisaccharide II was first recognized as the determinant of one of the I-antigens in man, I(Ma), but there is all probability that it has more fundamental significance, the I(Ma) antigen being expressed on the mouse embryos, from the single cell stage till after the sixth day of development.[6]

Material.- O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-D-galactose, I, was prepared by a known method.[7] Oligosaccharides O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-D-galactose III,[8] O-(β-D-galactopyranosyl)-(1→4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-D-galactose II,[9] and O-(β-D-galactopyranosyl)-(1→4)-O-(2-acetamido-2-deoxy-β-D-glucopyranoside)-(1→3)-D-galac-

tose IV,[9] were generous gifts of Dr A. Veyrières. Enzymes 2-5 (Table II) were commercial products. We ourselves isolated enzyme 1 (170 units from two litres of cow's colostrum) (cf.[10]). Ultrogel is a product of IBF, 92390 Villeneuve-la-Garenne (France).

Immobilization.- The enzyme (0.2 - 2 mg protein per mL of gel) was stirred at 4°C under N_2 for 14 h with Ultrogel activated by BrCN (60 mg per mL of gel). The gel was washed with M NaCl, bidistilled water and storage buffer, and stored at 4° under N_2 in the presence of substrates and 1 mM dithiothreitol. Immobilized activities were estimated by standard methods using suspensions, and the yield is expressed as 100 times the number of immobilized units over the total number utilized (Table).

Table. Optimized conditions for the preparation and storage of immobilized enzymes.

Enzyme	Immobilization buffer	Additives for immobilization and storage	Yield %	Storage buffer
1	(A)	UDPG, 1mM; GlcNAc, 5 mM; $MnCl_2$, 25 mM	24-34	(D)
2	(B)	UDPG, 0.5 mM; NAD, 5 mM	20-46	(E)
3	(C)	UDPG, 1.6 mM; PP_i, 1.6 mM	31	(F)
4	(C)	PP_i, 1 mM; P_i, 1 mM	41-51	(F)
5	(C)	ADP, 0.4 mM; PEP, 1 mM; $MgCl_2$, 10 mM	45-86	(F)

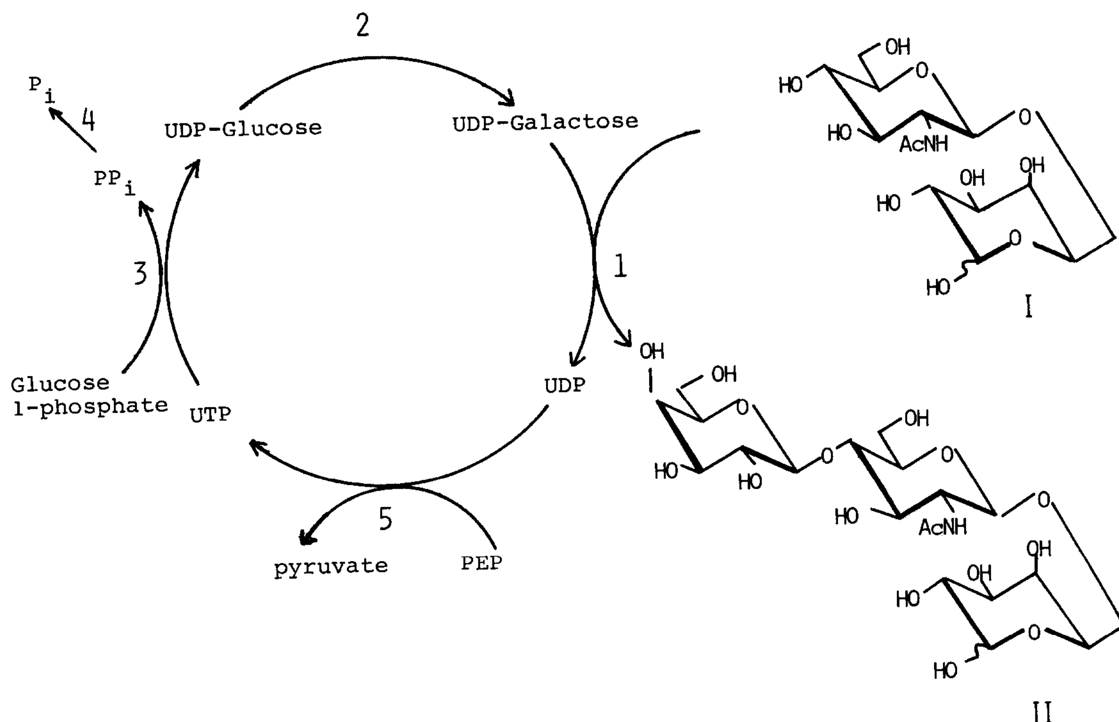
Buffers : (A), borate, 0.1 M; NaCl, 0.5 M; pH 8. (B), $NaHCO_3$, 0.1 M; NaCl, 0.5 M; pH 8.7. (C), $NaHCO_3$, 0.1 M, NaCl, 0.5 M, pH 8. (D), cacodylate, 25 mM, pH 7.4. (E), TRIS, 0.1 M; pH 8; (F), TRIS, 0.1 M; pH 7.5.

Galactosylation.- (Fig. 1). The pH of the reaction medium was kept in the near vicinity of 8 by the automatic delivery of either 50 mM HCl or 20 mM NaOH. The disaccharide (0.49 mmole), PEP (0.54 mmole), α-D-glucose-1-phosphate (0.54 mmole), $MnCl_2$ (0.10 mmole), $MgCl_2$ (0.20 mmole), KCl (3.5 mmole), dithiothreitol (0.5 mmole), UDPG (0.012 mmole), NAD (0.05 mmole), NaN_3 (5 mg) were dissolved in bidistilled water, the pH was ajusted to 8 and the enzymes were added : 1 (2.1 u); 2 (2.2 u); 3 (2.1 u), 4 (40 u); 5 (70 u). The total volume was 50 mL. The reaction was allowed to proceed at 30°C under N_2 in the gently shaken vessel until t.l.c. (isopropanol-ethyl acetate-water, 3:3:2) of a de-ionized aliquot indicated no more evolution. The gel was filtered, the solution was de-ionized, evaporated to dryness, and the trisaccharide isolated by Silica gel column chromatography.

Results and Discussion.

The immobilization of these five enzymes is a matter of about 4 days of work. All the components are inexpensive. After one galactosylation the mixture may be recovered by filtration, and used again several times with only a small loss of activity, for the galactosylation of either the same, or another

Figure 1. The operation of the β-D-galactopyranosylation cycle



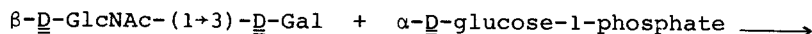
Enzymes : 1 : β-galactosyltransferase (E.C.2.4.1.22);
2 : UDP-galactose 4-epimerase (E.C.5.1.3.2); 3 : UDP-glucose pyrophosphorylase (E.C.2.7.7.9); 4 : inorganic pyrophosphatase (E.C.3.6.1.1); 5 : pyruvate kinase (E.C.2.7.1.40).

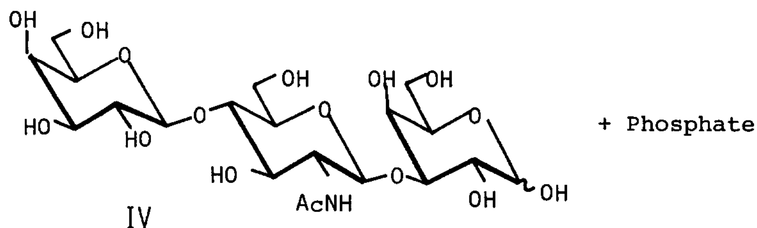
starting material. Altogether one milimole of galactosylation products were obtained with as little as 2 units of β-galactosyltransferase. It should be recalled that 170 units are easily prepared so that work on the hundredfold scale appears feasible. The bottlenecks in mixed processes will most probably appear in the chemical, not the enzymatic part.

All our reactions became very slow at about 70% of completion (see also [4]). At that limit, the turnover number for UTP was 28. The reaction lasts 2 days with disaccharide I and 5 days with disaccharide III. These are better substrates than free N-acetylglucosamine.[11] Some inhibitor may accumulate, which is, however, essentially removed in the recovery of the gel. Recycling of the sugar may achieve a practically complete conversion.

Figure 2 gives the stoichiometry of the glycosylation of III. Trisaccharides II and IV, both actually prepared on the 0.5 mmole scale, exhibited chromatographic behaviour and 250 MHz ^{1}H NMR spectra identical with those of authentic synthetic specimens.

Figure 2 : The stoichiometry of the galactosylation of III.





References and Notes.

1. H. Paulsen, Angew.Chem.Int.Ed.Engl., 21, 155 (1982).
2. The standard biochemical abbreviations have been used throughout this paper.
3. C. Augé and C. Gautheron, Colloque International sur les Réactifs Supportés, Lyon, Juin 1982.
4. C.H. Wong, S.L. Haynie and G.M. Whitesides, J.Org.Chem., 47, 5416 (1982).
5. A. Pollak, H. Blumenfeld, M. Wax, R.L. Baughn and G.M. Whitesides, J.Am.Chem.Soc., 102, 6324 (1980).
6. T. Feizi, Blood Transf.Immunohaematol., 23, 563-577 (1980).
7. C. Augé and A. Veyrières, J.C.S. Perkin I, 1343 (1977).
8. C. Augé and A. Veyrières, Carbohydr.Res., 54, 45 (1977).
9. C. Augé, S. David and A. Veyrières, Nouv.J.Chim., 3, 491 (1979).
10. R. Barker, K.W. Olsen, M. Shaper, and R.L. Hill, J.Biol.Chem., 247, 7135 (1972).
11. W.M. Blanken, G.J.M. Hooghwinkel and D.H. Van den Eijnden, Eur.J.Biochem., 127, 547 (1982).

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