Biochem. J. (1979) **177**, 569–574
Printed in Great Britain

The Assay of Xylosyltransferase in Cartilage Extracts

A MODIFIED PROCEDURE FOR PREPARATION OF SMITH-DEGRADED PROTEOGLYCAN

By JOHN D. SANDY
Department of Biochemistry, Monash University, Clayton, Vic. 3168, *Australia*

(*Received* 26 *June* 1978)

A modification of the published method [Baker, Rodén & Stoolmiller (1972) *J. Biol. Chem.* **247**, 3838–3847] for preparation of Smith-degraded proteoglycan is described. The new method is based on the finding that most of the chondroitin sulphate is cleaved from proteoglycan core protein by periodate oxidation. The borohydride reduction procedure was modified because the periodate-oxidized core protein is extensively degraded under the highly alkaline conditions previously used. The new method involves the separation of periodate-oxidized core protein from chondroitin sulphate by gel filtration on Sepharose 6B, and the reduction of the former in $H_3BO_3/NaBH_4$ at pH 8.5 to produce the reduced species. Smith-degraded proteoglycan prepared by this method exhibited high acceptor activity for xylosyltransferase from embryonic-chick cartilage and had an apparent K_m of 160 μg/ml or 45 μM on a serine basis. In this assay system an apparent K_m of 19 μM was obtained for UDP-xylose. The intermediate products periodate-oxidized core protein and reduced proteoglycan were inactive as xylosyltransferase acceptor substrates.

The linkage of chondroitin sulphate to the core protein of cartilage proteoglycan is through a glycosidic bond between xylose and serine (Rodén, 1970). The enzyme that catalyses the formation of this bond, and so initiates chondroitin sulphate synthesis, is xylosyltransferase (UDP-xylose–protein xylosyltransferase; EC 2.4.2.26). This enzyme was first detected in cell-free extracts of embryonic-chick cartilage by demonstrating the transfer of xylose from UDP-xylose to endogenous protein acceptors (Robinson *et al.*, 1966). Subsequently, it was shown that the enzyme could be assayed by incorporation of xylose into a number of exogenous serine-containing acceptors, the most active of which was Smith-degraded proteoglycan (Baker *et al.*, 1972).

The preparation of Smith-degraded proteoglycan from isolated proteoglycan has been described (Baker *et al.*, 1972; Rodén *et al.*, 1973). The method involves the sequential periodate oxidation, borohydride reduction and mild acid hydrolysis of proteoglycan; the procedure aims to remove chondroitin sulphate chains from the core protein under conditions that maintain protein structure and regenerate unsubstituted serine residues.

In initial attempts to prepare Smith-degraded proteoglycan by this method we obtained a degraded protein product that was inactive as a xylosyltransferase substrate. In an investigation of this problem we observed that most of the chondroitin sulphate was cleaved from the protein by periodate oxidation alone, and that the periodate-oxidized protein was degraded by borohydride treatment under the conditions previously used (Baker *et al.*, 1972). On the basis of these findings we have developed a modified method for preparation of Smith-degraded proteoglycan. The present paper gives details of the new method and describes studies made on the Smith-degraded proteoglycan and intermediate products of the procedure.

Experimental

Materials

Guanidine hydrochloride, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride, $NaBH_4$, UDP-xylose and Mes buffer (4-morpholine-ethanesulphonic acid) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. EDTA, CsCl, $NaClO_4$, $NaIO_4$ and H_3BO_3 were from BDH, Poole, Dorset, U.K. Benzamidine hydrochloride was from K & K Laboratories, Plainview, NY 11803, U.S.A. Enzyme-grade $(NH_4)_2SO_4$ was from Mann Research Laboratories, New York, NY 10006, U.S.A. UDP-[^{14}C]xylose (160 mCi/mmol) was from New England Nuclear, Boston, MA 02118, U.S.A. Sepharose 6B was from Pharmacia, Uppsala, Sweden.

Methods

Preparation of proteoglycan subunit. Proteoglycan subunit was prepared by a modification of a procedure described by Heinegard (1977). Bovine nasal septa, obtained fresh from the slaughterhouse, were finely sliced and extracted at 4°C for 48h in 10vol. of 4M-guanidine hydrochloride (containing 50mM-Tris/HCl buffer, pH7.5, 25mM-EDTA, 10mM-*N*-ethylmaleimide, 0.1mM-phenylmethanesulphonyl fluoride and 1mM-benzamidine hydrochloride). The extract was dialysed for 48h against 9vol. of 50mM-Tris/HCl buffer, pH7.5, and the dialysis residue was adjusted to 1.66g/ml with solid CsCl. Centrifugation under associative conditions was in an MSE-75 centrifuge at 40000rev./min for 40h at 10°C in an 8×25ml titanium angle rotor. The contents of the bottom one-third of each tube (A1 fractions) were combined, the guanidine hydrochloride concentration was adjusted to 4.0M and the density was adjusted to 1.60g/ml. Centrifugation under dissociative conditions was as described above for associative conditions, and the contents of the bottom two-sevenths of each tube (A1–D1 fractions) were combined and dialysed exhaustively against 3M-NaCl followed by dialysis for 72h against distilled water. The dialysis residue was freeze-dried, and the product (proteoglycan subunit) was stored at 20–25°C in a desiccator.

Preparation of xylosyltransferase. Enzyme was prepared from the epiphyseal cartilage of 14-day chick embryos as previously described (Stoolmiller *et al.*, 1972). The purification procedure was followed to Step V, and the 27–68%-satd.-$(NH_4)_2SO_4$ fraction was stored at 28mg of protein/ml and −20°C in 0.05M-Mes buffer, pH6.5, containing 0.2M-KCl, 12mM-$MgCl_2$, 3mM-$MnCl_2$ and 12.5mM-KF.

Assay of xylosyltransferase. The assay method was essentially that described previously (Stoolmiller *et al.*, 1972). Assays contained, in a total volume of 75μl, 0.89nmol (10nCi) of UDP-[^{14}C]xylose, 3.25μmol of Mes buffer, pH6.5, 13μmol of KCl, 0.78μmol of $MgCl_2$, 0.195μmol of $MnCl_2$, 0.81μmol of KF, 50–200μg of enzyme protein and 45μg of Smith-degraded proteoglycan. Incubation was at 37°C for 60 or 90min, and reactions were stopped by the addition of 0.5ml of 1% (w/v) bovine serum albumin and 0.15ml of 10% (w/v) trichloroacetic acid/4% (w/v) phosphotungstic acid followed by chilling to 0°C. The precipitate was collected by centrifugation, washed thoroughly three times in 0.3ml of 5% (w/v) trichloroacetic acid and dissolved in 0.1ml of 1M-NaOH for radioactivity measurement.

Analyses. Hexuronic acid was determined by a colorimetric method (Bitter & Muir, 1962), with glucuronolactone as standard. Interference by periodate was eliminated by reduction of the excess periodate with sorbitol. Protein was determined by a colorimetric method (Lowry *et al.*, 1951), with bovine serum albumin as standard. Hexosamines were determined after hydrolysis in 4M-HCl at 100°C for 8h. Total hexosamine was obtained by a colorimetric method (Boas, 1953), with glucosamine hydrochloride as standard. Galactosamine and glucosamine were separately determined in a Jeol amino acid analyser with corrections of 7 and 12% respectively for losses during hydrolysis (Swann & Balazs, 1966). Amino acids were determined in the Jeol analyser after hydrolysis in constant-boiling HCl at 110°C for 24h *in vacuo*. Radioactivity was measured by addition of aqueous samples (2ml) to 8ml of toluene/Triton X-114 (1:1, v/v), containing (per litre) 8g of 2,5-diphenyloxazole, and counting in a Philips automatic liquid-scintillation analyser. Periodate was determined by dilution of reaction mixtures in water and measurement of $A_{222.5}$ as previously described (Crouthamel *et al.*, 1949).

Results and Discussion

In initial attempts to prepare Smith-degraded proteoglycan by the published method (Baker *et al.*, 1972) we obtained a degraded core-protein product that was inactive as an acceptor substrate for xylosyltransferase in extracts of chick embryonic cartilage. To investigate this problem we decided to carry out each of the preparative steps (periodate oxidation, borohydride reduction and mild acid hydrolysis) individually and thereby isolate the intermediate products of the procedure.

Periodate oxidation of proteoglycan

Proteoglycan subunit (205mg) was oxidized with 0.5M-$NaIO_4$ in 0.25M-$NaClO_4$ as previously described (Baker *et al.*, 1972). The course of the reaction was followed by measuring the consumption of periodate and glucuronic acid over a 6-day period (Fig. 1). It is seen that during the first 72h, 66% (140μmol) of the glucuronic acid was oxidized with the consumption of 600μmol of periodate. This consumption of 4.3mol of periodate/mol of glucuronic acid oxidized indicates that under these reaction conditions 'over-oxidation' of the polysaccharide occurred (see Bobbitt, 1956; Lindberg *et al.*, 1975). 'Over-oxidation', which implies oxidative reactions other than 1,2-glycol cleavage, is also indicated by the continued consumption of periodate in the 96–144h period when glucuronic acid oxidation has apparently ceased. The 'over-oxidation' of chondroitin sulphate would indeed be expected under these conditions (Bobbitt, 1956), since an approximately 10-fold molar excess of periodate over oxidizable substrate was used, and the reaction was performed at room temperature without stringent

precautions to exclude oxygen or light from the reaction mixture.

The products after 72h of reaction were isolated by fractionation on Sepharose 6B (Fig. 2). Periodate oxidation under these conditions resulted in the separation of most of the chondroitin sulphate from proteoglycan protein. Thus the core protein, in association with small amounts of hexosamine and hexuronic acid, was recovered in fractions 18–28, and the chondroitin sulphate fragments released were recovered in fractions 30–37. The analysis of the periodate-oxidized core protein isolated in this way (Table 1) shows that the periodate treatment removes chondroitin sulphate, but not keratan sulphate, from the core protein. Thus whereas the galactosamine/total amino acid ratio is markedly lower in periodate-oxidized core protein than in proteoglycan subunit, the glucosamine/total amino acid ratio is essentially the same in these two preparations.

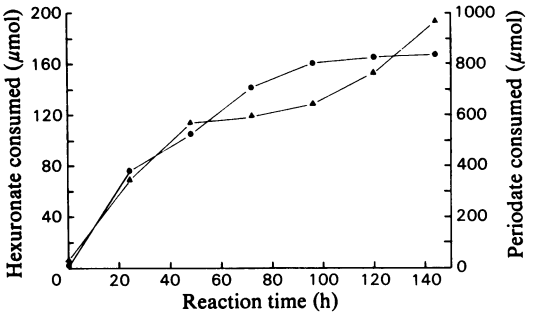

Fig. 1. *Periodate oxidation of proteoglycan subunit*
Proteoglycan subunit (205mg) was dissolved in 45ml of 0.25M-$NaClO_4$ in the dark at 22–25°C; 5ml of 0.5M-$NaIO_4$ was added and the consumption of periodate (▲) and hexuronic acid (●) was followed with time.

The site(s) of cleavage of chondroitin sulphate during oxidation are unknown. The average molecular weights of chondroitin sulphate prepared by either alkaline β-elimination or by periodate treatment of proteoglycan subunit were determined on a calibrated Sephadex G-200 column (Hopwood & Robinson, 1973) and found to be 23000 and 6000 respectively. This is consistent with periodate causing three or four cleavages of the chondroitin sulphate chains. One cleavage site is apparently near the linkage to the core protein, since more than 90% of the galactosamine was removed from the core protein on oxidation (Fig. 2).

Degradation of proteoglycan chondroitin sulphate during periodate oxidation has previously been observed by Scott (1976), and a mechanism for the periodate-dependent cleavage of acetal- and ether-linked polymers has been proposed (Scott & Tigwell, 1973). It is based on disproportionation reactions induced by hydroxyl radicals generated in periodate solutions in the presence of light. Polymer cleavage

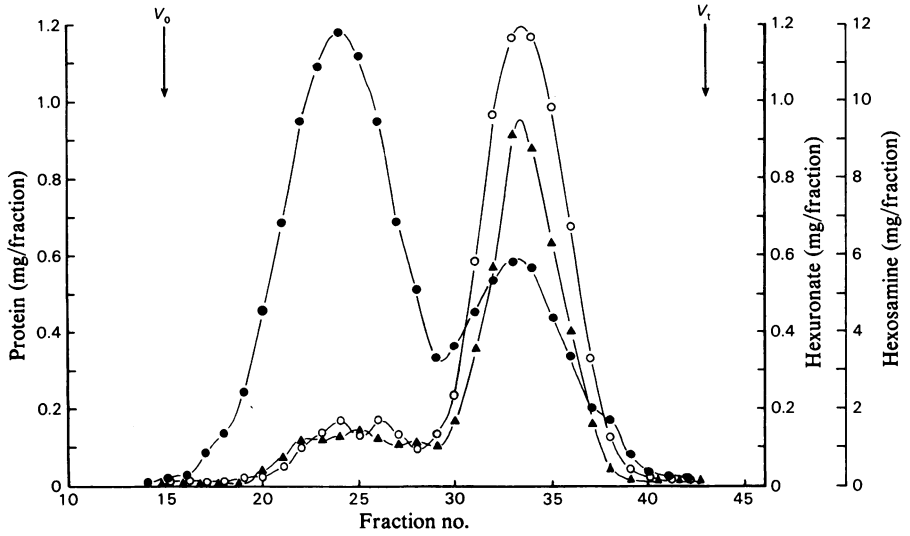

Fig. 2. *Fractionation of periodate-oxidized proteoglycan on Sepharose 6B*
Proteoglycan subunit (205mg) was oxidized for 72h with $NaIO_4$ (see the text for conditions), the product was dialysed exhaustively against water, freeze-dried, dissolved in 0.2M-NaCl and fractionated on a column (60cm×3cm) of Sepharose 6B which was eluted at 24ml/h with 0.2M-NaCl. Fractions (about 12ml) were analysed for protein (●), hexuronic acid (○) and hexosamine (▲).

Table 1. *Analytical values for products obtained in the preparation of Smith-degraded proteoglycan*
See the text for details. Abbreviations: PGS, proteoglycan subunit; PGPO, periodate-oxidized proteoglycan; PGSD, Smith-degraded proteoglycan.

Fraction	Amount of fraction (mg)	Protein (mg)	Serine (μmol)	Galactosamine/total amino acid (molar ratio)	Glucosamine/total amino acid (molar ratio)
PGS	205	10.4	13.53	3.6	0.20
PGPO	38.2	7.8	8.12	0.72	0.17
PGSD	25.4	6.7	7.21	0.89	0.20

apparently results from radical-catalysed opening of the pyranoid ring between C-1 and the ring oxygen followed by C-2–C-3 glycol scission. It appears that the α-hydroxy acid structure of the glycuronans renders them particularly susceptible to such degradation, as evidenced by rapid viscosity changes on periodate treatment of alginate (Scott *et al.*, 1976). It seems likely therefore that the cleavage of chondroitin sulphate observed in the present work results from radical-induced scission of a limited number of glucuronic acid residues in the glycosaminoglycan chain.

Smith degradation of periodate-oxidized core protein

When periodate-oxidized core protein isolated from Sepharose 6B (Fig. 2) was treated with $NaBH_4$ under the conditions previously used (Baker *et al.*, 1972), and the reduction products were again fractionated on the Sepharose 6B column, the core protein was extensively degraded. Thus the high-molecular-weight protein species (fractions 18–28, Fig. 2) was no longer present, and protein fragments were recovered over a broad fractionation range between fraction 25 and fraction 40.

This degradation of protein was due to the highly alkaline conditions (pH > 12.5) that occur in 0.15% (w/v) borohydride solutions in the absence of buffer. Thus when the reduction was performed in Na_3BO_3 buffer at pH 8.5 (Spiro, 1964), a high-molecular-weight protein product (reduced proteoglycan) was obtained which exhibited essentially the same fractionation properties on Sepharose 6B as the periodate-oxidized core protein. The susceptibility of proteoglycan core protein to degradation in solutions above pH 11 has been described by Knight (1976) in studies on the alkaline β-elimination of chondroitin sulphate from proteoglycan. Further, the exposure of the periodate-oxidized core protein to unbuffered borohydride might be expected to result in an inactive xylosyltransferase substrate as a result of alkaline β-elimination of xylosyl–serine bonds and the conversion of substituted serine residues into dehydroalanine.

The third step in Smith-degraded proteoglycan preparation, that is the mild acid hydrolysis of reduced proteoglycan, was carried out as previously described (Baker *et al.*, 1972), with the period of hydrolysis extended to 72h. The core protein appears to be quite stable to this treatment, since the product (Smith-degraded proteoglycan; Table 1) was of a molecular-weight range similar to periodate-oxidized core protein and reduced proteoglycan, as determined by Sepharose 6B chromatography.

On the basis of the work described above, the following modified procedure for preparation of Smith-degraded proteoglycan was developed.

The modified procedure for preparation of Smith-degraded proteoglycan

Proteoglycan subunit (0.2g) was dissolved in 45ml of 0.25M-$NaClO_4$ at 22–25°C in the dark. $NaIO_4$ (0.5M; 5ml) was added with mixing, and the solution (initial pH 5.0) was maintained between pH 4.5 and pH 5.0 by addition of KOH. After 72h the reaction mixture was dialysed against water at 4°C for 16h, and the dialysis residue was freeze-dried. The dried material was dissolved in 10ml of 0.2M-NaCl and was applied to a column (60cm × 3cm) of Sepharose 6B, which was eluted at 24ml/h with 0.2M-NaCl (Fig. 2). Fractions 18–28, which contained the high-molecular-weight protein, were combined and dialysed against water at 4°C for 16h, and the dialysis residue was freeze-dried (periodate-oxidized proteoglycan). The dried periodate-oxidized core protein was dissolved in 30ml of 0.1M-H_3BO_3, and $NaBH_4$ (0.21g) was added slowly with mixing at 22–25°C. The solution (initial pH 9.2) was adjusted to pH 8.5 with HCl and stored at 4°C for 16h. During this period the reaction mixture remained between pH 8.5 and pH 9.2 without further adjustment. The reduction was terminated by the addition of acetic acid to pH 5.0 and dialysis against water at 4°C for 16h. The dialysis residue (reduced proteoglycan) was adjusted to pH 1 by careful addition, with mixing, of HCl, and acid hydrolysis was allowed for 72h at 22–25°C. This solution was carefully adjusted to pH 7.0 with KOH, dialysed exhaustively against water at 4°C and freeze-dried. The yield of product (Smith-degraded proteoglycan) was generally about 25mg.

Analytical values obtained for proteoglycan subunit, periodate-oxidized core protein and Smith-

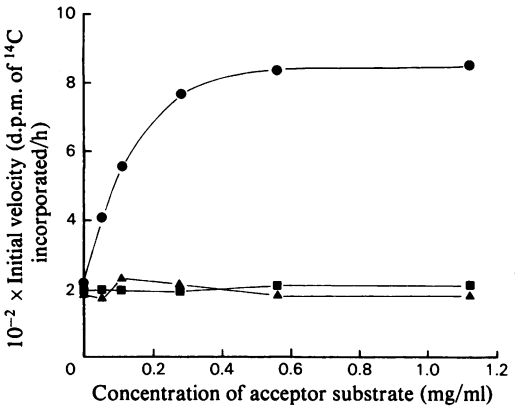

Fig. 3. *Determination of the acceptor substrate activity of periodate-oxidized core protein, reduced proteoglycan and Smith-degraded proteoglycan*

Incubations contained, in a total volume of 75 μl, 0.89 nmol (10 μCi) of UDP-[^{14}C]xylose, 3.25 μmol of Mes buffer, pH 6.5, 13 μmol of KCl, 0.78 μmol of $MgCl_2$, 0.195 μmol of $MnCl_2$, 0.81 μmol of KF, partially purified xylosyltransferase from embryonic-chick cartilage (140 μg/assay) and various concentrations of periodate-oxidized core protein (▲), reduced proteoglycan (■) and Smith-degraded proteoglycan (●). Incubation was at 37°C for 60 min, and the trichloracetic acid-insoluble products were isolated (see under 'Methods') for radioactivity measurement.

degraded proteoglycan in a typical preparation are shown in Table 1. The yield of protein or serine in this procedure was generally between 55 and 65%. It is apparent from the hexosamine analyses that the effect of the Smith degradation is to remove chondroitin sulphate but not keratan sulphate from the core protein. Thus the molar ratio of galactosamine to total amino acid decreases from 3.6 in proteoglycan subunit to 0.89 in Smith-degraded proteoglycan, whereas that of glucosamine to total amino acid is essentially unaltered. The serine content of the Smith-degraded proteoglycan prepared by this method (0.285 μmol/mg) is very similar to that of Smith-degraded proteoglycan prepared by Baker *et al.* (1972) (0.30 μmol/mg).

Xylosyltransferase studies

Smith-degraded proteoglycan and the intermediate products of the procedure described above (periodate-oxidized core protein and reduced proteoglycan) were tested as acceptor substrates for xylosyltransferase (Fig. 3). The enzyme used in these studies was a partially purified preparation from embryonic-chick epiphyseal cartilage (see under 'Methods'). Although Smith-degraded proteoglycan exhibited high acceptor activity, the intermediate products were inactive over the concentration range tested. The apparent K_m for Smith-degraded proteoglycan, determined by a double-reciprocal plot of these data, was 160 μg/ml or 45 μM on a serine basis. These values are similar to those obtained by Baker *et al.* (1972) for their Smith-degraded proteoglycan preparation. In the standard assay, Smith-degraded proteoglycan was added at a concentration of 0.6 mg/ml and under these conditions the incorporation of [^{14}C]xylose into the trichloroacetic acid-insoluble product was linear with time over at least 90 min of incubation.

Smith-degraded proteoglycan prepared by the new method exhibited high acceptor activity, as indicated by the rate of xylose transfer to this substrate by partially purified (Step V) enzyme from embryonic-chick cartilage (4.26 nmol/h per mg of Smith-degraded proteoglycan per mg of enzyme protein). With a similar enzyme preparation but using their Smith-degraded proteoglycan sample, Stoolmiller *et al.* (1972) obtained a transfer rate of about 3.5 nmol/h per mg of Smith-degraded proteoglycan per mg of enzyme protein. Smith-degraded proteoglycan prepared by the new method has also been used to assay xylosyltransferase in homogenates of cultured embryonic-chick chondrocytes and in a soluble fraction prepared from both adult and foetal bovine articular cartilage (J. D. Sandy, unpublished observations).

The finding that periodate-oxidized core protein and reduced proteoglycan were inactive as acceptor substrates (Fig. 3) is consistent with the suggestion (Baker *et al.*, 1972) that only specific serine residues of the core protein can act as xylose acceptors. Both

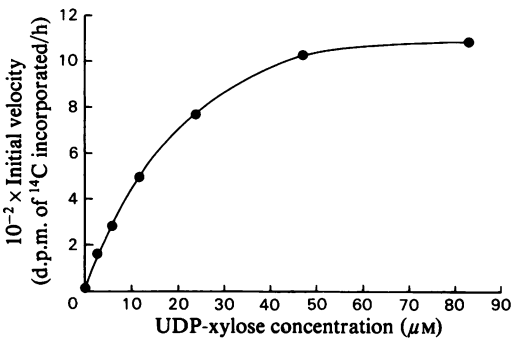

Fig. 4. *Effect of UDP-xylose concentration on xylosyltransferase activity*

Incubations contained, in a total volume of 75 μl, 3.25 μmol of Mes buffer, pH 6.5, 13 μmol of KCl, 0.78 μmol of $MgCl_2$, 0.195 μmol of $MnCl_2$, 0.81 μmol of KF, partially purified xylosyltransferase from embryonic-chick cartilage (140 μg/assay), 45 μg of Smith-degraded proteoglycan, and various concentrations of UDP-[^{14}C]xylose (sp. radioactivity 11.2 μCi/μmol). Incubation was at 37°C for 90 min, and the trichloroacetic acid-insoluble products were isolated (see under 'Methods') for radioactivity measurement.

periodate-oxidized core protein and reduced proteoglycan are essentially free of chondroitin sulphate (Fig. 2); however, it appears that the xylosyl–serine bond of the chondroitin sulphate linkage region is still intact in these preparations. The presence of this bond in periodate-oxidized core protein and reduced proteoglycan is indeed expected from the reactions involved in the Smith degradation (Bobbitt, 1956). Thus in periodate-oxidized core protein the xylose probably exists as the dialdehyde and in reduced proteoglycan as the dialcohol. It is only after acid hydrolysis of the acyclic acetal linkage of the dialcohol that the xylosyl–serine bond is expected to be cleaved and the serine hydroxyl group regenerated.

The effect of UDP-xylose concentration on reaction velocity is shown in Fig. 4. A double-reciprocal plot of these data yielded an apparent K_m for UDP-xylose of 19μM. An apparent K_m for UDP-xylose of 25μM was previously observed with a similar enzyme preparation from embryonic chick (Stoolmiller *et al.*, 1972) and a value of 10μM was obtained with highly purified xylosyltransferase from rat chondrosarcoma (Schwartz & Dorfman, 1975).

This work was supported by the Australian Research Grants Committee (Project No. 74-1500IR). I thank Dr. H. C. Robinson and Professor D. A. Lowther for helpful discussion and Heather L. G. Brown for skilled technical assistance.

References

Baker, J. R., Rodén, L. & Stoolmiller, A. C. (1972) *J. Biol. Chem.* **247**, 3838–3847

Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334

Boas, N. F. (1953) *J. Biol. Chem.* **204**, 553–563

Bobbitt, J. M. (1956) *Adv. Carbohydr. Chem. Biochem.* **11**, 1–41

Crouthamel, C. E., Meek, H. V., Martin, D. S. & Banks, C. V. (1949) *J. Am. Chem. Soc.* **71**, 3031–3035

Heinegard, D. (1977) *J. Biol. Chem.* **252**, 1980–1989

Hopwood, J. J. & Robinson, H. C. (1973) *Biochem. J.* **135**, 631–637

Knight, K. (1976) Ph.D. Dissertation, Monash University

Lindberg, B., Loungren, J. & Svensson, S. (1975) *Adv. Carbohydr. Chem. Biochem.* **31**, 185–240

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Robinson, H. C., Telser, A. & Dorfman, A. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1859–1866

Rodén, L. (1970) in *Metabolic Conjugation and Metabolic Hydrolysis* (Fishman, W. H., ed.), vol. 2, pp. 345–442, Academic Press, New York

Rodén, L., Baker, J. R., Helting, T., Schwartz, N. B., Stoolmiller, A. C., Yamagata, S. & Yamagata, T. (1973) *Methods Enzymol.* **28**, 638–676

Schwartz, N. B. & Dorfman, A. (1975) *Arch. Biochim. Biophys.* **171**, 136–144

Scott, J. E. (1976) *Front. Matrix Biol.* **3**, 176–191

Scott, J. E. & Tigwell, M. J. (1973) *Carbohydr. Res.* **28**, 53–59

Scott, J. E., Tigwell, M. J., Phelps, C. F. & Nieduszynski, I. A. (1976) *Carbohydr. Res.* **47**, 105–117

Spiro, R. G. (1964) *J. Biol. Chem.* **239**, 567–573

Stoolmiller, A. C., Horwitz, A. L. & Dorfman, A. (1972) *J. Biol. Chem.* **247**, 3525–3532

Swann, D. A. & Balazs, E. A. (1966) *Biochim. Biophys. Acta* **130**, 112–129