Biochem. J. (1970) **120**, 719–723
Printed in Great Britain

Biosynthesis of Glycosaminoglycans in Bovine Cornea

THE EFFECT OF URIDINE DIPHOSPHATE XYLOSE

By C. BALDUINI, A. BROVELLI and A. A. CASTELLANI
Department of Biological Chemistry, Faculty of Science, University of Pavia, 27100 *Pavia, Italy*

(*Received* 9 *June* 1970)

1. The role of UDP-xylose in the regulation of corneal glycosaminoglycan biosynthesis was investigated. Bovine corneas were incubated with [U-^{14}C]-glucose in the presence and in the absence of the nucleotide, and the radioactivity of chondroitin, chondroitin sulphate and keratan sulphate, as well as of their monosaccharide constituents, was determined. 2. A decrease in the rate of biosynthesis of chondroitin and chondroitin sulphate and an increase in that of keratan sulphate were observed in the samples incubated with UDP-xylose. 3. The UDP-glucuronic acid isolated after the incubation in the presence of UDP-xylose showed a noticeable decrease in the amount of radioactivity incorporated; this result suggests that UDP-xylose inhibits the UDP-glucose dehydrogenase, causing an accumulation of UDP-glucose and consequently an increase in the formation of UDP-galactose and keratan sulphate. 4. Galactose and galactosamine isolated from the polysaccharides showed variations in the amount of radioactivity incorporated in accordance with those observed for the macromolecules; this fact confirms that in the system we used *in vitro* a real biosynthesis of the polysaccharide chain took place and that the regulatory effect of UDP-xylose was active at the monosaccharide level.

Xylose has been found in chondromucoprotein (Castellani, Bonferoni, Ronchi, Ferri & Malcovati, 1962) and plays an important role in linking the carbohydrate chains to the protein moiety of the molecule (Rodén, 1965, 1968); it is present in the linkage region also in other glycosaminoglycans (Lindahl & Rodén, 1965; Fransson, 1968). Xylose can be synthesized in cartilage (Castellani, Calatroni & Righetti, 1967) from UDP-glucuronic acid by a UDP-glucuronate decarboxylase, previously demonstrated in plant tissues (Feingold, Neufeld & Hassid, 1960). The UDP-xylose that is synthesized by this decarboxylase is probably a precursor of chondromucoprotein, since a xylosyltransferase has been demonstrated in cartilage by Robinson, Telser & Dorfman (1966) and by Telser, Robinson & Dorfman (1966). Neufeld & Hall (1965) reported that UDP-xylose is a specific inhibitor of UDP-glucose dehydrogenase and suggested for this nucleotide a probable role as a regulator in the biosynthesis of chondroitin sulphate. In the present work we examined this hypothesis by studying the effect of UDP-xylose on the biosynthesis *in vitro* of glycosaminoglycans (chondroitin, chondroitin sulphate and keratan sulphate) of bovine cornea.

EXPERIMENTAL

Extraction of corneas. The corneas were dissected from eyes that were removed immediately after the death of the calf and put in Krebs–Ringer phosphate buffer [0.154 M-NaCl–0.154 M-KCl–0.11 M-$CaCl_2$–0.154 M-$MgSO_4$–0.1 M-Na_2HPO_4 adjusted to pH 7.4 with 1 M-HCl (100:4:3:1:20, by vol.)] and cooled (to 4°C) for about 1 h until their arrival in our laboratory; 40 corneas were usually collected.

Incubation of corneas. The corneas were cleaned, cut along a diameter and the two halves were put in different vessels. Krebs–Ringer phosphate buffer (10 ml), 1–5 μmol of each amino acid (Robert & Parlebas, 1965), 10 μCi of [U-^{14}C]glucose and 1–5 μmol of ATP were added to each pool prepared in this way; 0.5 mM-UDP-xylose was added to one of the two pools. The corneas were incubated at 37°C for different times in a shaking bath (Robert & Parlebas, 1965). After the incubation the corneas were washed with 0.9% NaCl.

Extraction and separation of glycosaminoglycans. The digestion with papain (Sigma) of the corneas and the separation of glycosaminoglycans on Dowex 1 (X2) were done as described by Mathews & Cifonelli (1965).

Extraction of UDP-glucuronic acid. Corneas that had been incubated, under the above conditions, for 3 h were washed with 0.9% NaCl and water and finally homogenized in 3% (w/v) trichloroacetic acid. All operations

after the incubation were carried out at 4°C. Carrier UDP-glucuronic acid (2mg) was added to the homogenate, which was then centrifuged at 18000*g*. The pellet was again homogenized in trichloroacetic acid and centrifuged. The supernatants were pooled and repeatedly shaken in a separatory funnel with 1 vol. of diethyl ether to eliminate trichloroacetic acid (10–12 extractions). They were then dried with a Rotovapor apparatus, and the residue was dissolved in water. The solution was filtered on a Bio-Gel P-2 column (10cm × 1cm) and dried again.

The separation of UDP-glucuronic acid was carried out with the Hitachi–Perkin–Elmer chromatography apparatus, on a column (10cm × 0.9cm) of Dowex 1 (X4; Cl^- form). A gradient of NaCl was used to elute the nucleotide. The gradient was obtained with three vessels respectively containing 100ml of 0.01M-HCl, 100ml of 0.05M-NaCl in 0.01M-HCl and 200ml of 0.25M-NaCl in 0.01M-HCl. The column was equilibrated at 30°C and the flow rate was 120ml/h. The UDP-glucuronic acid peak was collected and desalted on Dowex 50 (X8). The glucuronic acid content and the incorporated radioactivity in the aqueous eluate were determined.

Preparative t.l.c. of galactose. The isolated keratan sulphate–peptide was hydrolysed in 1M-HCl for 120min at 105°C in a sealed tube under N_2. The galactose was recovered by t.l.c. on Kieselgel G plates, with butanol–acetic acid–water (6:3:1, by vol.) as solvent.

Preparative ion-exchange chromatography of galactosamine. The chondroitin sulphate–peptide was hydrolysed in 4M-HCl for 7h in a sealed tube under N_2 at 105°C. Galactosamine was separated on an Hitachi spherical-resin No. 2610 column (15cm × 0.9cm) by using the Hitachi–Perkin–Elmer chromatography apparatus in standard conditions; the recovered peak was desalted on a Dowex 50 (X8) column, which was washed with water and eluted with 0.5M-NH_3.

Determination of radioactivity. The samples, weighed on a Cahn electrobalance, were dissolved in 0.6ml of water, and 6ml of ethanol and 8.4ml of toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene were added (Perlman, Telser & Dorfman, 1964). For the determination of radioactivity a Packard Tri-Carb liquid-scintillation spectrometer was used.

RESULTS

Role of UDP-xylose on the incorporation of [U-^{14}C]-*glucose into corneal glycosaminoglycans.* The results obtained after an incubation of 4h are summarized in Table 1. The presence of UDP-xylose in the incubation medium produced a significant decrease in the radioactivity incorporated from glucose into the chondroitin and chondroitin sulphate, and a proportional increase in the radioactivity of keratan sulphate. It was noted that in the absence of UDP-xylose the radioactivity incorporated into chondroitin and chondroitin sulphate was always higher than that into keratan sulphate. Variation in the specific radioactivities of the glycosaminoglycans is evident by reference to Table 1; this was due to the difficulty of exactly standardizing the time between the death of the animals and the beginning of the incubation.

Fig. 1 shows the rate of incorporation of radioactivity into chondroitin, chondroitin sulphate and

Table 1. *Specific radioactivity of glycosaminoglycans from bovine corneas*

Bovine corneas were incubated for 4h with [U-^{14}C]glucose in the absence and in the presence of 0.5mM-UDP-xylose. The glycosaminoglycans were fractionated on Dowex 1 (X2) with different concentrations of NaCl (Mathews & Cifonelli, 1965).

	Specific radioactivity of glycosaminoglycan		
	(c.p.m./mg)		% difference
Glycosaminoglycan	Without UDP-xylose	With UDP-xylose	
Chondroitin (1M-NaCl)			
Expt. 1	1750	1145	−34.6
Expt. 2	2351	2067	−12.0
Expt. 3	1860	1127	−39.4
Expt. 4	2380	2220	−6.7
Chondroitin sulphate (1.5M-NaCl)			
Expt. 1	2492	1780	−28.5
Expt. 2	3042	2405	−20.9
Expt. 3	1452	1188	−18.2
Expt. 4	5110	5042	−1.3
Keratan sulphate (3M-NaCl)			
Expt. 1	1095	1357	+23.9
Expt. 2	726	879	+21.0
Expt. 3	831	1011	+21.6
Expt. 4	1283	1754	+36.7

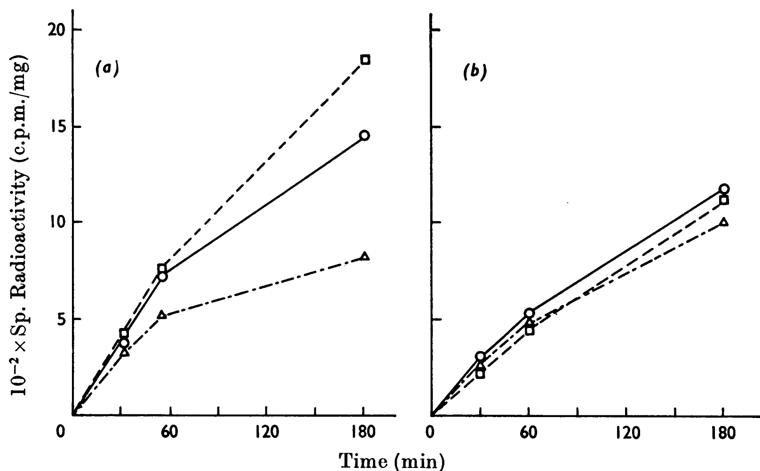

Fig. 1. Biosynthesis of glycosaminoglycans in bovine cornea. Bovine corneas were incubated for different times with [U-^{14}C]glucose in the absence (*a*) and in the presence (*b*) of 0.5mM-UDP-xylose. The figure shows the variation of the rate of biosynthesis induced by this nucleotide in chondroitin (□), chondroitin sulphate (○) and keratan sulphate (△).

Table 2. *Specific radioactivity of UDP-glucuronic acid from bovine corneas*

UDP-glucuronic acid was isolated after incubation of bovine corneas for 3h with [U-^{14}C]glucose in the absence and in the presence of 0.5mM-UDP-xylose. Carrier UDP-glucuronic acid (2mg) was added before the extraction of the nucleotide. In Expt. 2 the corneas were preincubated for 30min with UDP-xylose before the [U-^{14}C]-glucose was added.

	Specific radioactivity of UDP-glucuronic acid		
	(c.p.m./mg)		% difference
	Without UDP-xylose	With UDP-xylose	
Expt. 1	14265	9886	−30.7
Expt. 2	18850	8720	−53.7

keratan sulphate in the absence and in the presence of UDP-xylose. There was no difference in the radioactivity of the three polysaccharides for periods of incubation up to about 30min, and the reasons for this are not understood. If the incubation was continued longer than this it is seen that UDP-xylose caused a marked decrease in the rate of incorporation of label into chondroitin and chondroitin sulphate and an increase in the rate of its incorporation into keratan sulphate. The presence of the nucleotide thus caused the specific radioactivities of the three macromolecules to be alike.

Role of UDP-xylose in the incorporation of [U-^{14}C]*glucose into UDP-glucuronic acid.* Table 2 shows that the action exerted by UDP-xylose on the biosynthesis of glycosaminoglycans is also observed in the formation of UDP-glucuronic acid. In fact the presence of UDP-xylose in the incubation medium produced a remarkable decrease of the radioactivity incorporated into the nucleotide precursor in the biosynthesis of chondroitin and chondroitin sulphate. This decrease was more evident when corneas were preincubated with UDP-xylose (Expt. 2) before the addition of [U-^{14}C]glucose.

Determination of radioactivity in some monosaccharide units isolated from keratan sulphate–peptide and chondroitin sulphate–peptide. To demonstrate that biosynthesis of the carbohydrate chains *de novo* occurred in our experimental conditions, we tested the radioactivity of galactose as a parameter of keratan sulphate biosynthesis and of galactosamine as a parameter of chondroitin sulphate biosynthesis. Galactose isolated from keratan sulphate–peptide extracted from corneas incubated with UDP-xylose was more radioactive than galactose isolated from the keratan sulphate–peptide of the standard corneas (Table 3).

Similarly UDP-xylose induced a decrease in the

Table 3. *Specific radioactivity of monosaccharides isolated from cornea glycosaminoglycans*

Galactose and galactosamine were prepared by hydrolysis of glycosaminoglycans of which the radioactivity was reported in Table 1.

	Specific radioactivity of monosaccharide		
	(c.p.m./mg)		% difference
Monosaccharide	Without UDP-xylose	With UDP-xylose	
Galactose			
Expt. 2	1466	1814	+23.7
Expt. 4	3076	4487	+45.8
Galactosamine			
Expt. 2	3027	2031	−32.9

radioactivity of the galactosamine of chondroitin sulphate–peptide.

It is noteworthy that galactose contained a higher radioactivity than the other monosaccharide component of the macromolecule. The specific radioactivity of galactosamine showed that this sugar must be less labelled than glucuronic acid. This result might be explained if the hexosamine biosynthesis from glucose required several steps for which a dilution of the labelled precursor may be postulated.

DISCUSSION

The results obtained clearly suggest that in the system we used *in vitro* UDP-xylose can regulate the biosynthesis of corneal glycosaminoglycans. This action consists of an inhibition in the synthesis of the chondroitin and chondroitin sulphate polysaccharidic chains and of an increase in the synthesis of the keratan sulphate–peptide chain. The mechanism involved in this regulation is represented by the inhibition exerted by UDP-xylose on UDP-glucose dehydrogenase, as reported by Neufeld & Hall (1965) and proved in our experiments by the fact that UDP-xylose induced a decrease in the synthesis of UDP-glucuronic acid from [U-^{14}C]-glucose. Probably this decrease leads to an accumulation of UDP-glucose, which can be isomerized to UDP-galactose, and can in this way stimulate the synthesis of keratan sulphate. Alternatively it seems possible that the increased keratan sulphate biosynthesis could be due to a stimulation of UDP-glucose epimerase by a decrease of the [NADH]/[NAD$^+$] ratio, produced by the UDP-glucose dehydrogenase inhibition. The increased biosynthesis of keratan sulphate seems to be confirmed by the presence in galactose of most of the radioactivity of keratan sulphate, and by the presence in the monosaccharides of the same incorporation change induced by UDP-xylose and observed in the experiments in which the radioactivity of the macromolecule was determined.

The relevant problem is whether the regulatory mechanism observed *in vitro* has any physiological significance. There are several considerations suggesting that the above-mentioned mechanism could take place also *in vivo*. We know that in cartilage the quantitative ratio between chondroitin sulphate and keratan sulphate undergoes variations with aging similar to the ones induced *in vitro* by UDP-xylose (Kaplan & Meyer, 1959; Mathews, 1967; Pedrini & Pedrini-Mille, 1968), and it was also proved (Castellani *et al.* 1967) that UDP-xylose can be synthesized in cartilage from UDP-glucuronic acid. An increase in the activity of UDP-glucuronate decarboxylase during aging could produce an increase of UDP-xylose and hence regulate the biosynthesis of glycosaminoglycans. Thus xylose is a component of the protein–polysaccharide linkage of chondromucoprotein, for which UDP-xylose is a precursor (Robinson *et al.* 1966). An accumulation of UDP-xylose could be also due to a decrease in the activity of the xylosyltransferase. In this case the UDP-xylose may reach such a concentration in the cell that it could have the regulatory effect mentioned. This hypothesis is supported by the suggestion by Horwitz & Dorfman (1968) that the transferase, which links the first sugar to the protein core, acts on the rough endoplasmic reticulum. If the sites of protein synthesis and of xylosyltransferase action are contiguous then it seems likely that a variation in transferase activity could in some way control the synthesis of the protein moiety. These hypotheses must be examined further to interpret fully the effect of UDP-xylose on the biosynthesis of glycosaminoglycans.

Aureli, Rizzotti, Balduini & Castellani (1969) observed, by histochemical techniques, that UDP-glucose dehydrogenase of cartilage is considerably decreased in older animals. A similar decrease was observed when cartilage of young animals was incubated with UDP-xylose, and these results also suggest that the nucleotide can get into the cell or can at any rate exert its effect intracellularly. It is

generally accepted that nucleotides do not enter the cell; however, the present results indicated that UDP-xylose added to medium had a regulatory role on UDP-glucose dehydrogenase activity. We do not know whether this nucleotide entered the corneal cell and thus played a direct role or if it regulated the enzyme activity by some more complex mechanism.

We are grateful to Professor A. Dorfman (University of Chicago) for helpful suggestions given to us for this research. This work was supported by funds from Consiglio Nazionale delle Ricerche, Italy.

REFERENCES

Aureli, G., Rizzotti, M., Balduini, C. & Castellani, A. A. (1969). *Riv. Istochem. norm. Patol.* **15**, 9.

Castellani, A. A., Bonferoni, B., Ronchi, S., Ferri, G. & Malcovati, M. (1962). *Ital. J. Biochem.* **11**, 187.

Castellani, A. A., Calatroni, A. & Righetti, P. G. (1967). *Ital. J. Biochem.* **16**, 5.

Feingold, D. S., Neufeld, E. F. & Hassid, W. Z. (1960). *J. biol. Chem.* **235**, 910.

Fransson, L. A. (1968). *Biochim. biophys. Acta*, **156**, 311.

Horwitz, A. & Dorfman, A. (1968). *J. Cell Biol.* **38**, 358.

Kaplan, D. & Meyer, K. (1959). *Nature, Lond.*, **183**, 1267.

Lindahl, U. & Rodén, L. (1965). *J. biol. Chem.* **240**, 2821.

Mathews, M. B. (1967). *Biol. Rev.* **42**, 499.

Mathews, M. B. & Cifonelli, J. A. (1965). *J. biol. Chem.* **240**, 4140.

Neufeld, E. F. & Hall, C. W. (1965). *Biochem. biophys. Res. Commun.* **19**, 456.

Pedrini, V. & Pedrini-Mille, A. (1968). In *The Chemical Physiology of Mucopolysaccharides*, p. 139. Ed. by Quintarelli, G. Boston: Little, Brown and Co.

Perlman, R. L., Telser, A. & Dorfman, A. (1964). *J. biol. Chem.* **239**, 3623.

Robert, L. & Parlebas, J. (1965). *Bull. Soc. Chim. biol.* **47**, 1853.

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

Rodén, L. (1965). *Structure and Function of Connective and Skeletal Tissue*, p. 141. London: Butterworth and Co. Ltd.

Rodén, L. (1968). In *The Chemical Physiology of Mucopolysaccharides*, p. 17. Ed. by Quintarelli, G. Boston: Little, Brown and Co.

Telser, A., Robinson, H. C. & Dorfman, A. (1966). *Fedn Proc. Fedn Am. Socs exp. Biol.* **25**, 588.