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Uridine Diphosphate Glucose Dehydrogenase from Cornea and Epiphysial-Plate Cartilage

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1. UDP-glucose dehydrogenase (EC 1.1.1.22) was extracted from epiphysial-plate cartilage of newborn pigs and from whole bovine corneas. 2. Formation of UDP-glucuronic acid was demonstrated by radioautography after separation of the sugar nucleotides by paper chromatography or t.l.c.: in these conditions a radioactive glucuronic acid spot also appears. 3. UDP-xylose prevented the formation in the incubation mixture of both UDP-glucuronic acid and free glucuronic acid. 4. In both tissues the dependence of the enzyme activity on pH and the K_m values for UDP-glucose and NAD^+ were determined. 5. Inhibition by UDP-xylose with respect to UDP-glucose was investigated. The plots of $1/v$ versus 1/[UDP-glucose], and of percentage inhibition versus UDP-xylose concentration and the Hill coefficient showed that a co-operative effect existed between UDP-xylose-binding sites. 6. The physiological meaning of the different affinities of cartilage and cornea enzymes for UDP-xylose is discussed and related to the different glycosaminoglycan contents of the two connective tissues studied.

The UDP-glucose dehydrogenase reaction was first described in calf liver (Dutton & Storey, 1951; Strominger *et al.*, 1954) and in epiphysial-plate cartilage (Castellani *et al.*, 1957). More recently this enzyme has been studied in plants (Neufeld & Hall, 1965), yeasts (Ankel *et al.*, 1966), bacteria (Bdolah & Feingold, 1968*a*) and animal tissues (Neufeld & Hall, 1965; Bdolah & Feingold, 1968*b*; Gainey & Phelps, 1972), and it was demonstrated that all these UDP-glucose dehydrogenases are strongly and specifically inhibited by UDP-xylose with a feedback mechanism. In cornea and cartilage UDP-glucuronic acid is directly utilized for the biosynthesis of chondroitin sulphate or is transformed into UDP-xylose, which is the first sugar nucleotide involved in the biosynthesis of the linkage region (Grebner *et al.*, 1966*a*,*b*; Robinson *et al.*, 1966; Telser *et al.*, 1966). In recent years in our laboratory the effect of UDP-xylose on glycosaminoglycan biosynthesis in bovine cornea and newborn-pig epiphysial-plate cartilage has been investigated. After incubation of cornea slices with [U-^{14}C]glucose and UDP-xylose, a decrease in chondroitin sulphate synthesis and a concomitant increase in keratan sulphate synthesis have been observed (Balduini *et al.*, 1970). We have interpreted these results as an inhibition of UDP-glucose dehydrogenase, with a decrease in UDP-glucuronic acid and therefore in chondroitin sulphate synthesis. The consequent accumulation of UDP-glucose, or the variation in NAD^+/NADH ratio, might increase the UDP-galactose 4′-epimerase activity and therefore the biosynthesis of keratan sulphate. In the same experimental conditions this effect is not observed with newborn-pig epiphysial-plate cartilage slices; in this case UDP-xylose at different concentrations induces only a small increase in chondroitin sulphate biosynthesis (C. Balduini & G. De Luca, unpublished work). To determine whether the differences observed in the experiments with the two tissues *in vitro* can be ascribed to a diversity in the characteristics of the two UDP-glucose dehydrogenases, we have studied in the present paper kinetic parameters and in particular the effect of UDP-xylose on the enzyme isolated from whole bovine cornea and newborn-pig epiphysial-plate cartilage.

Experimental

Extraction of UDP-glucose dehydrogenase from newborn-pig epiphysial-plate cartilage

Epiphysial-plate cartilages were removed from legs of newborn-pigs just after the death and bleeding of the animals. For each experiment three or four animals were killed, from which 4 or 5g of cartilage was obtained. The excised cartilage was kept at 4°C in 0.15M-KCl containing 10mM-2-(*N*-morpholino)-ethanesulphonic acid, 1mM-EDTA and 0.1mM-dithiothreitol. The tissue was then homogenized with

10–12.5ml (2.5vol.) of the same solution, with an Ultra-Turrax homogenizer, until all the cartilage fragments were transformed into a viscous material. Great care was taken in maintaining the temperature during homogenization at 4–5°C. The homogenate was then centrifuged in a Spinco L2 50B preparative ultracentrifuge for 40min at 4°C and 87000*g*. The supernatant was utilized for all the experiments. When incubated in the assay conditions described below, 1ml of enzyme preparation usually produced 10–14nmol of UDP-glucuronic acid/min.

Extraction of bovine cornea UDP-glucose dehydrogenase

Corneas were excised directly in the slaughterhouse just after the death of the calf, placed in Krebs–Ringer phosphate buffer [0.154M-NaCl–0.154M-KCl–0.11M-$CaCl_2$–0.154M-$MgSO_4$–0.1M-Na_2HPO_4, adjusted to pH7.4 with 1M-HCl (100:4:3:1:20, by vol.)] and cooled to 4°C for about 1h until arrival in the laboratory; 12–16 corneas (6–8g) was the number usually collected. After repeated washings the whole corneas were homogenized in 12–16ml (2vol.) of 10mM-2-(*N*-morpholino)ethanesulphonic acid, 1mM-EDTA, 0.1mM-dithiothreitol, or simply in double-distilled water, the temperature being kept at 4°C. The last procedure was preferably used because it resulted in a better recovery of the enzyme, whose kinetic characteristics were unmodified. The homogenate was mechanically stirred for 3h at 4°C. Potassium chloride was then added to a concentration of 0.15M; the homogenate was centrifuged at 4°C for 40min at 87000*g* and the supernatant was then used for all the experiments. When incubated in the assay conditions described below, 1ml of enzyme preparation usually produced 4–7nmol of UDP-glucuronic acid/min.

Enzyme assay

Two different methods were used to assay UDP-glucose dehydrogenase activity.

(1) Reduction of NAD^+ was followed at 37°C in a 0.8ml-capacity 1cm-path-length cuvette containing, in the standard assay, 1mM-UDP-glucose, 1mM-NAD^+, 200–300μl of the enzyme preparation and Tris–HCl buffer (Tris concentration 0.025M) to a volume of 0.8ml at pH8.8. The reaction was started by the addition of the enzyme after preincubation of the mixture; the increase in E_{340} was measured in a Saitron spectrophotometer (Florence, Italy). The initial rate was recorded by using a full-scale deflexion of 0.1 E_{340} unit.

(2) UDP-glucuronic acid formation was determined by Bitter & Muir's (1962) uronic acid assay, after incubation of the enzyme preparation with the substrates. Incubation was carried out at 37°C for 70min for the cartilage enzyme and for 90min for the cornea enzyme. During this time the reaction rate shows only a slight fall from linearity; shorter incubation periods result in not very reliable uronic acid assays. The composition of the mixture in the standard assay was the same as that reported above. In all the experiments a control was prepared, having the same composition but with the enzyme heat-inactivated. After incubation, the interfering substances (uronic acid-containing glycosaminoglycans originally present in the enzyme preparation) were removed by precipitation with 99% ethanol, after the addition of sodium acetate and acetic acid to concentrations of 5% (w/v) and 0.5M respectively. The supernatant, containing UDP-glucuronic acid, resulting from a centrifugation for 15min at 3000*g* was used in the uronic acid assay. This second method was used to confirm the results obtained with the first one; a slight reduction of NAD^+ also occurred in the absence of UDP-glucose, so that it was necessary to correct the E_{340} variations recorded in our experiments. However, the results obtained with the two assay methods were in agreement. Enzyme activity was expressed as nmol of UDP-glucuronic acid produced/min by 1ml of enzyme preparation.

Kinetic analysis

The K_m for UDP-glucose was determined, with 1mM-NAD^+, by varying the UDP-glucose concentration from 0.03 to 1.7mM. The K_m for NAD^+, with 1mM-UDP-glucose, was studied at NAD^+ concentrations from 0.1 to 0.9mM.

In the inhibition experiments, the dependence of reaction velocity on UDP-glucose and NAD^+ concentrations was studied in the presence of UDP-xylose concentrations from 0.01 to 0.12mM.

Identification of reaction products

The UDP-glucuronic acid formed by the UDP-glucose dehydrogenase reaction was identified after incubation of the enzyme preparation with 2mM-UDP-[U-^{14}C]glucose (1μCi/μmol), 1mM-NAD^+ and Tris–HCl buffer (Tris concentration 0.025M), in a final volume of 250μl at 37°C for 90min. After incubation the enzyme was heat-inactivated at 100°C for 3min and the sugar nucleotides were separated by (A) paper chromatography or (B) t.l.c.

(A) Descending paper chromatography was performed on Whatman 3MM paper strips in propan-2-ol–1% (w/v) $(NH_4)_2SO_4$ (2:1, v/v). The chromatogram was run for 30h (Hall & Khorana, 1954). (B) T.l.c. was carried out on 5% (w/v) polyethyleneimine-impregnated MN 300 cellulose plates (100μm thick) (Machery, Nagel and Co., Düren, Germany) (Cohn & Segal, 1969). The solvent was 0.2M-LiCl

and the plates were run for 6h at 4°C. The appropriate standards were included in each run. Non-radioactive sugar nucleotides were detected under u.v. light. Non-radioactive reducing sugars were located by the aniline hydrogen phthalate spray (Waldi, 1965). Radioactive products were detected by radioautography, by using Kodak Regulix films, with an exposure time of 15 days. Distribution of radioactivity was measured with a Beckman LS-230 liquid-scintillation system directly on 1 cm-long paper strips cut along the direction of chromatography. The scintillation solution had the following composition: 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene dissolved in toluene.

Results

Formation of UDP-glucuronic acid and glucuronic acid

The enzyme preparation extracted from pig epiphysial-plate cartilage, when incubated with UDP-[U-^{14}C]glucose, produces radioactive UDP-glucuronic acid: radioautography and determination of the distribution of radioactivity on the paper chromatograms obtained for the experiments without UDP-xylose show a radioactive spot corresponding to the position of standard UDP-glucuronic acid (Fig. 1). It is noteworthy that the same incubation mixture also contains free radioactive glucuronic acid. This evidence is in agreement with the hypothesis of Gainey & Phelps (1972), who indicated the presence in cartilage of a phosphatase activity acting on the glucuronic acid 1-phosphate produced by a nucleotidase from UDP-glucuronic acid. Fig. 1 also shows that the presence in the incubation mixture of 2mM-UDP-xylose inhibits the formation of both UDP-glucuronic acid and free glucuronic acid. The same results were obtained when the cornea enzyme was used; radioautography of the t.l.c. plates showed the formation of radioactive UDP-glucuronic acid, which does not appear when 2mM-UDP-xylose is added to the incubation mixture.

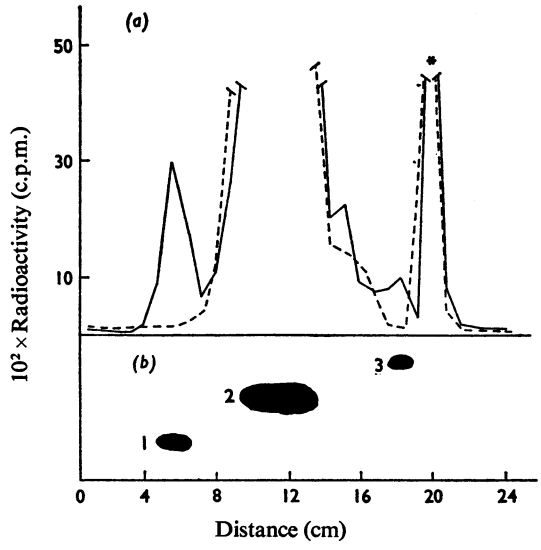

Fig. 1. *Identification of the product of the cartilage UDP-glucose dehydrogenase reaction in the presence and absence of UDP-xylose*

Reaction mixtures contained 2mM-[U-^{14}C]glucose, 1mM-NAD^+ and 250μl of enzyme extract in Tris-HCl buffer, pH8.8 (Tris concentration 0.025M). The incubation was carried out at 37°C for 90min. The reaction mixture was then run on Whatman 3MM chromatographic paper and eluted for 30h with propan-2-ol-1% (w/v) $(NH_4)_2SO_4$ (2:1, v/v). Together with the samples the appropriate standards were run. Non-radioactive UDP-glucose and UDP-glucuronic acid were located under u.v. light; glucuronic acid was detected by the aniline hydrogen phthalate spray. (*a*) Radioactivity of chromatogram: ——, sample incubated without UDP-xylose; – – –, sample incubated with 2mM-UDP-xylose; *, unidentified peak. (*b*) Standards: 1, UDP-glucuronic acid; 2, UDP-glucose; 3, glucuronic acid.

pH optimum and effect of substrate concentration

Optimum pH. The pH optimum was determined for the cartilage enzyme by using Tris-HCl buffer (Tris concentration 0.025M) between pH7.3 and 9.0, and glycine-NaOH buffer (0.1M-glycine and 0.1M-NaOH appropriately mixed) between pH8.5 and 11.0. As shown in Fig. 2, the optimum is in a range between pH8.8 and 9.0. A maximum of activity, which we are not able to explain, also appears at pH9.8–10.0.

The pH optimum for the cornea enzyme was determined with 0.1M-sodium phosphate buffer between pH6.0 and 8.0, with Tris-HCl buffer (Tris concentration 0.025M) between pH8.0 and 9.4, and with glycine-NaOH buffer (0.1M-glycine and 0.1M-NaOH appropriately mixed) between pH8.4 and 10.6. In this case, too, optimum pH is in the range 8.8–9.0, and a maximum of activity is also present at pH9.8–10.0.

Substrate concentration. The K_m values of cartilage and cornea UDP-glucose dehydrogenases for UDP-glucose and for NAD^+, determined at 37°C by Lineweaver & Burk (1934) plots, are reported in Table 1.

Inhibition by UDP-xylose

The inhibitory effect of UDP-xylose on both cartilage and cornea UDP-glucose dehydrogenases has been indicated by radioautographic evidence

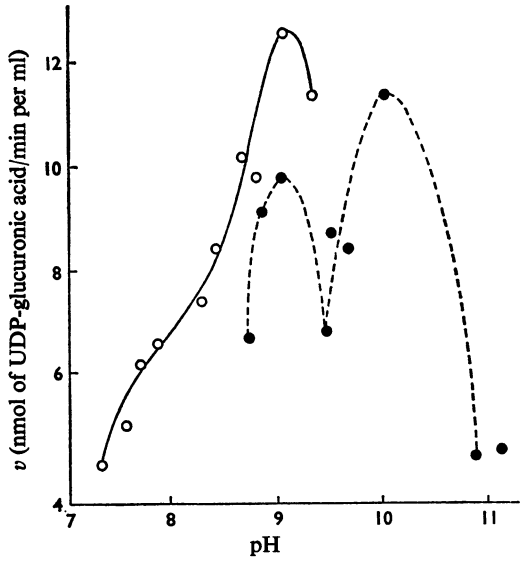

Fig. 2. *Effect of pH on cartilage UDP-glucose dehydrogenase activity*

Reaction velocity (v) is expressed as nmol of UDP-glucuronic acid produced/min by 1 ml of enzyme preparation. For experimental details see the text. ○, Tris–HCl buffer (Tris concentration 0.025 M); ●, glycine–NaOH buffer (0.1 M-glycine and 0.1 M-NaOH appropriately mixed).

Table 1. *K_m values of cartilage and corneal UDP-glucose dehydrogenase*

For experimental details see the text.

Source of the enzyme	K_m M	
	UDP-glucose	NAD^+
Pig epiphysial-plate cartilage	3.5×10^{-4}	3.0×10^{-4}
Bovine cornea	2.0×10^{-3}	1.3×10^{-3}

(Fig. 1). The kinetic study of the inhibition indicates that this sugar nucleotide acts as an allosteric inhibitor of the enzyme from epiphysial-plate cartilage and cornea. Figs. 3(*a*) and 3(*b*) show that the reciprocal plots of reaction velocity versus UDP-glucose concentration, in the presence of various UDP-xylose concentrations, depart progressively from linearity with increasing UDP-xylose concentration and yield a family of curves that show an upward inflexion. This effect is more evident for low UDP-glucose and high UDP-xylose concentrations.

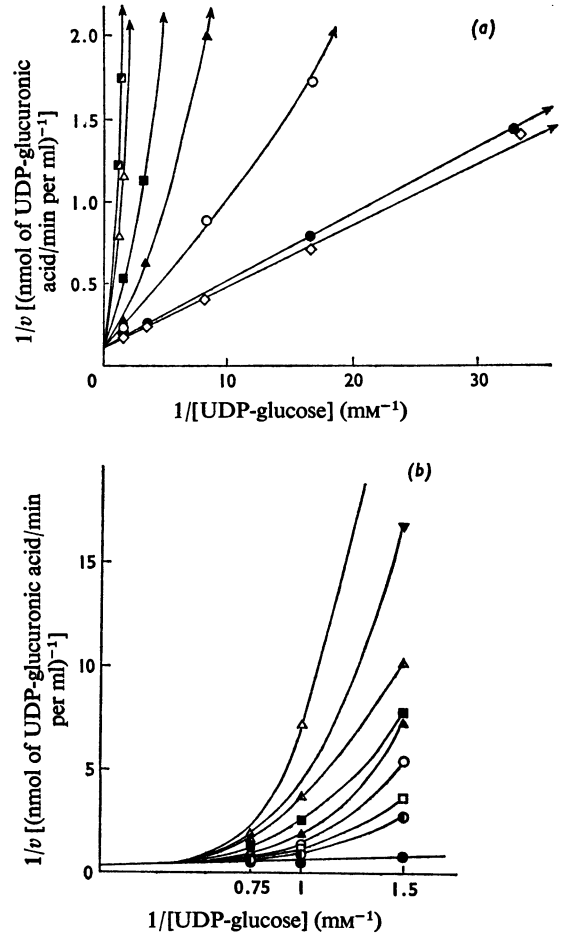

Fig. 3. *Reciprocal plots of velocity versus UDP-glucose concentration at constant saturating NAD^+ concentrations and various concentrations of UDP-xylose, for UDP-glucose dehydrogenase from (a) epiphysial-plate cartilage and (b) cornea*

Reaction velocity (v) is expressed as nmol of UDP-glucuronic acid produced/min by 1 ml of enzyme preparation. For experimental details see the text. UDP-xylose concentrations (mM): (*a*) cartilage: ◇, 0; ●, 0.01; ○, 0.02; ▲, 0.03; ■, 0.06; △, 0.09; ◩, 0.12; (*b*) cornea: ●, 0; ◐, 0.009; □, 0.012; ○, 0.024; ▲, 0.035; ■, 0.047; ▲, 0.055; ▼, 0.074; △, 0.091.

Plots of percentage inhibition versus UDP-xylose concentration at different UDP-glucose concentrations (Figs. 4*a* and 4*b*) result in curves that, both for cartilage and cornea enzyme, are sigmoidal. The Hill coefficient values graphically estimated by plotting $\log[(V-v)/v]$ versus log[UDP-xylose] (Taketa & Pogell, 1965) are 1.4 for the enzyme from cartilage and

1.25 for the enzyme from cornea. These results suggest that co-operative interactions exist between several inhibitor-binding sites on the enzyme (Monod *et al.*, 1963).

As shown in Figs. 4(*a*) and 4(*b*), the UDP-xylose concentration necessary to obtain a 50% inhibition of the enzyme for the same UDP-glucose concentration is much higher for the UDP-glucose dehydrogenase from cartilage than for the enzyme from cornea; if we consider, for example, a UDP-glucose concentration of about 1 mM the UDP-xylose concentration giving a 50% inhibition is 0.008 mM for the enzyme from cornea and 0.045 mM for the enzyme from cartilage. This result indicates significant difference in the properties of the two enzymes and is discussed below.

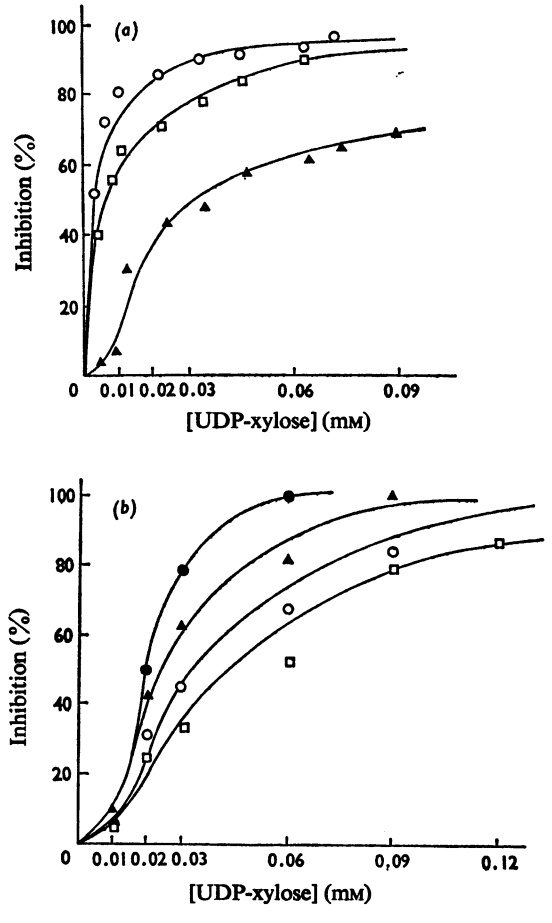

Fig. 4. *Percentage inhibition of the UDP-glucose dehydrogenase activity at various UDP-glucose and UDP-xylose concentrations in (a) cornea and (b) cartilage*

UDP-glucose concentration in the reaction mixtures was (*a*), for corneal enzyme: ○, 0.67 mM; □, 1 mM; ▲, 1.33 mM; (*b*), for cartilage enzyme: ●, 0.12 mM; ▲, 0.30 mM; ○, 0.60 mM; □, 0.90 mM. Percentage inhibition is defined as 100 × (activity in the absence of UDP-xylose − activity in the presence of UDP-xylose)/activity in the absence of UDP-xylose. The assay conditions are those described in the text, except for the UDP-glucose concentrations.

Discussion

UDP-glucose dehydrogenases extracted from whole bovine cornea and newborn-pig epiphysial-plate cartilage and their regulation by UDP-xylose have been studied in the present paper. The two UDP-glucose dehydrogenases have the same optimum pH of 8.8–9.0, similar to that reported for the enzyme from other sources (Gainey & Phelps, 1972; Molz & Danishefsky, 1971; Bdolah & Feingold, 1968*b*; Neufeld & Hall, 1965).

The K_m values of the cartilage enzyme are 3.5×10^{-4} M for UDP-glucose and 3.0×10^{-4} M for NAD^+. The K_m values for the cornea enzyme are 2.0×10^{-3} M for UDP-glucose and 1.3×10^{-3} M for NAD^+. The UDP-glucose dehydrogenase from cartilage shows a higher affinity for both the substrates (about five times): it is noteworthy that UDP-glucose cellular concentration in rat epiphysial cartilage is about one-quarter that in corneal epithelium and stroma (Handley & Phelps, 1972*a*,*b*). It is remarkable that K_m values reported by Gainey & Phelps (1972) for corneal epithelium enzyme are lower than the values we obtained. This is probably attributable to the fact that we extracted whole cornea, which is composed of endothelium, epithelium and stroma, whereas Gainey & Phelps (1972) used only epithelium.

The effect of UDP-xylose on both the enzymes is similar to that reported for UDP-glucose dehydrogenase from other sources: pea seedlings and bovine liver (Neufeld & Hall, 1965), neonatal skin, bovine corneal epithelium, sheep nasal-septum cartilage (Gainey & Phelps, 1972), hen oviduct (Bdolah & Feingold, 1968*b*), *Cryptococcus laurentii* (Ankel *et al.*, 1966), rat liver (Molz & Danishefsky, 1971), chick-embryo liver and eye (Darrow & Hendrickson, 1971). The sugar nucleotide acts as a co-operative allosteric inhibitor as proved by the upward inflexion of the plots of $1/v$ versus 1/[UDP-glucose]; this inflexion is particularly evident for low UDP-glucose and high UDP-xylose concentrations. This conclusion is further substantiated by the sigmoid nature of the inhibition curves and by the Hill coefficients ($n = 1.4$ for the enzyme from cartilage and $n = 1.25$ for the enzyme from cornea). However, the quantitative determinations of UDP-xylose inhibition of the two enzymes show significant differences. The amount of UDP-xylose necessary to produce a 50% inhibition is

five to six times higher for UDP-glucose dehydrogenase from epiphysial-plate cartilage than for the enzyme from cornea. The recent papers of C. J. Handley and C. F. Phelps on the concentration of the sugar nucleotides in rat epiphysial cartilage (Handley & Phelps, 1972*a*) and bovine cornea (Handley & Phelps, 1972*b*,*c*) allow speculation about the physiological meaning of these differences. In fact if we plot percentage inhibition versus UDP-xylose concentration for the two enzymes, at UDP-glucose concentrations similar to physiological cellular values (about 0.4mM in corneal stroma, 0.3mM in corneal epithelium and 0.1mM in rat epiphysial cartilage), the results reported in Fig. 5 are obtained. The enzyme from pig epiphysial-plate cartilage, with 0.12mM-UDP-glucose, is only 14% inhibited in the presence of 0.013mM-UDP-xylose (physiological cellular concentrations for rat epiphysial cartilage) (Handley & Phelps, 1972*a*). The enzyme from whole cornea, in the presence of 0.6mM-UDP-glucose (the concentration used in our experiments, which is a little higher than the physiological one) and of 0.070mM-UDP-xylose (the physiological concentration in corneal stroma; Handley & Phelps, 1972*b*), is more than 90% inhibited.

These results can be explained in terms of the spectrum of glycosaminoglycans produced by the two tissues that we have studied; bovine cornea produces both chondroitin sulphate and keratan sulphate whereas newborn-pig epiphysial-plate cartilage produces mostly chondroitin sulphate, only small traces of keratan sulphate being present (Castellani *et al.*, 1962). It is therefore obvious that, considering the slow turnover rate of keratan sulphate in cornea (Handley & Phelps, 1972*c*), the UDP-glucose dehydrogenase in this tissue is almost inactive so as to allow UDP-glucose utilization in keratan sulphate biosynthesis. In epiphysial-plate cartilage, however, chondroitin sulphate biosynthesis is very rapid (Handley & Phelps, 1972*a*), so that the enzyme activity must be kept high. These results are consistent with our previous results on the biosynthesis of glycosaminoglycans in cornea (Balduini *et al.*, 1970) and epiphysial-plate cartilage slices, which indicated that UDP-xylose induced a significant decrease in chondroitin sulphate synthesis in cornea, although it had no effect on chondroitin sulphate biosynthesis in cartilage slices. The diverse affinities of UDP-glucose dehydrogenase extracted from cornea and epiphysial-plate cartilage for UDP-xylose could explain these differences. However, other mechanisms may exist that can interact with UDP-xylose inhibition to regulate UDP-glucose utilization in biosynthetic pathways of glycosaminoglycans in different tissues. The idea that xylosyltransferase, with the characteristic of dissociating and reassociating to the multi-glycosyltransferase complex, can play a regulatory role in biosynthesis of proteoglycans (Stoolmiller *et al.*, 1972) is of great interest. In our experiment with epiphysial-plate cartilage a high xylosyltransferase activity might prevent UDP-xylose accumulation and consequently its inhibitory effect. On the other hand a low xylosyltransferase activity in cornea could explain the high UDP-xylose concentration and consequently the strong physiological inhibition of UDP-glucose dehydrogenase in this tissue.

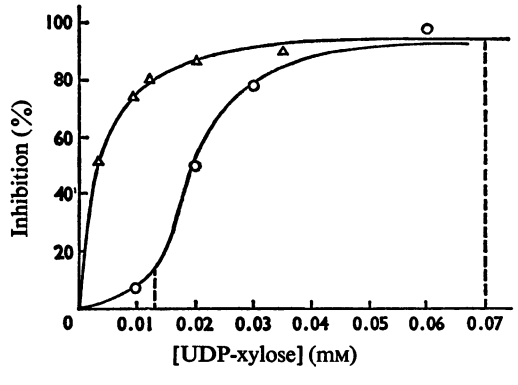

Fig. 5. *Inhibition of cartilage and cornea UDP-glucose dehydrogenase activity at physiological concentrations of UDP-glucose and UDP-xylose*

For details see the text. ○, Pig epiphysial-plate cartilage UDP-glucose dehydrogenase with 0.12mM-UDP-glucose; △, cornea UDP-glucose dehydrogenase with 0.60mM-UDP-glucose. Broken lines indicate the percentage inhibition corresponding to the physiological concentrations of UDP-xylose in epiphysial cartilage (0.013mM) and cornea (0.070mM).

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