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Uridine Diphosphate Glucuronic Acid Production and Utilization in Various Tissues Actively Synthesizing Glycosaminoglycans

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1. UDP-glucose dehydrogenase has been partially purified from sheep nasal septum cartilage, neonatal rat skin and bovine corneal epithelium. 2. The pH profile, K_m values for NAD^+ and UDP-glucose, activation energy and molecular weight have been determined for the enzyme from several of the tissues. 3. The sugar nucleotide concentrations in each of the tissues have been related to the spectrum of glycosaminoglycans produced by each tissue. 4. The presence of an allosteric UDP-xylose-binding site distinct from the active site(s) in sheep nasal septum UDP-glucose dehydrogenase has been demonstrated. 5. An active UDP-glucuronic acid nucleotidase has been demonstrated in sheep nasal cartilage. 6. Tissue-space experiments have shown the cell water content of sheep nasal septum cartilage to be 14% of the wet weight. 7. Glucuronic acid 1-phosphate does not occur in measurable amounts in sheep nasal septum cartilage and no UDP-glucuronic acid pyrophosphorylase activity could be detected in this tissue. 8. The inhibition by UDP-xylose with respect to both substrates, UDP-glucose and NAD^+, has been examined, and shown to be allosteric.

UDP-glucuronic acid donates the glucuronic acid moiety of acidic mucopolysaccharides and of glucuronides in detoxification and in the production of ascorbic acid (Dutton, 1966). UDP-glucuronic acid may also be utilized in the production of UDP-galacturonic acid in bacteria and plants (Feingold *et al.*, 1960; Smith *et al.*, 1958), UDP-iduronic acid in animals (Jacobson & Davidson, 1962) and UDP-xylose in plants and animals (Ankel & Feingold, 1965; Bdolah & Feingold, 1965). Each of these compounds are variously utilized in polysaccharide biosynthesis.

In animals and plants UDP-glucuronic acid is produced from UDP-glucose by the action of UDP-glucose dehydrogenase (EC 1.1.1.22). This enzyme has been obtained from various sources and is specifically and potently inhibited by UDP-xylose in an allosteric manner (Neufeld & Hall, 1965).

In the present paper we establish the rate of synthesis of UDP-glucuronic acid in three tissues, each of which produces a different spectrum of glycosaminoglycans, and we relate the observed sugar nucleotide concentrations to the kinetic parameters of UDP-glucose dehydrogenase isolated from each of the tissues. In particular the effect of UDP-xylose on UDP-glucose dehydrogenase has been studied in sheep nasal septum cartilage, bovine cornea, and neonatal rat skin, where the rates of utilization of UDP-glucuronic acid by the detoxification and ascorbate pathways are presumably small.

Materials and Methods

Materials

Chemicals were obtained from the following: Norit A (acid washed) from Pfanstiehl Laboratories Inc., Waukegan, Ill., U.S.A. UDP-D-[U-^{14}C]-glucuronic acid (ammonium salt, specific radioactivity 287mCi/mmol), [U-^{14}C]sucrose (specific radioactivity 9.6mCi/mmole), ^3H_2O (specific radioactivity 1mCi/ml) from The Radiochemical Centre, Amersham, Bucks., U.K.; 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen from Thorn Electronics Ltd., Tolworth, Surrey, U.K.; Whatman 3MM chromatography paper and Whatman GF/B glass paper from H. Reeve Angel and Co. Ltd., London E.C.4, U.K; Sephadex G-200 from Pharmacia, Uppsala, Sweden; Dowex-1 (Cl^- form) from Dow Chemical International S.A., Midland, Mich., U.S.A.; Zeo-Karb 225, from BDH Chemicals Ltd., Poole, Dorset, U.K.; NAD^+ (free salt), NADH, UDP-glucose (sodium salt) and catalase from Boehringer Corporation (London) Ltd., London W.5, U.K.; myoglobin, UDP-xylose, bovine serum albumin (fraction V), inorganic pyrophosphatase and mercaptoethanol from Sigma Chemical Corporation, St. Louis, Mo., U.S.A.; trimethylchlorosilane and hexamethyldisilazane from Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flints., U.K.

All other chemicals used were AnalaR or the equivalent grade where possible. Dialysis tubing was purchased from Visking Union Carbide, Chicago, Ill., U.S.A., and was boiled for 3–6h with three changes of double-distilled water before use.

Methods

Centrifugation. Unless otherwise stated centrifugation was done at 4°C in an MSE High-Speed 18 centrifuge with an 8 × 50ml head (type 69181).

Measurement of radioactivity. Water-based samples of up to 0.3ml in volume were counted for radioactivity in low-background glass bottles containing 8ml of scintillation fluid [toluene–2-methoxyethanol (3:2, v/v) containing 80g of naphthalene and 4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen/l] in a Packard Tri-Carb model 3320 liquid-scintillation system. An external standard was used to correct for quenching in the samples. Simultaneous measurements of ^{14}C and ^{3}H radioactivity was achieved by suitable discrimination in the above machine.

Uronic acid assay. The presence of uronic acid was demonstrated by the method of Bitter & Muir (1962).

Paper chromatography. Descending paper chromatography of nucleotide sugars and sugar phosphates was done by using Whatman 3MM chromatography paper previously washed with 1% oxalic acid. The chromatograms were eluted with 1M-ammonium acetate buffer (pH3.8)–ethanol mixture, pH4.8 (2:5, v/v), for 22h as described by Paladini & Leloir (1952). The appropriate standards were included on each run.

Homogenization. Homogenization was done with a variable-speed Omnimix top-drive homogenizer (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) in a stainless-steel sample holder.

Ammonium sulphate fractionation. Solid ammonium sulphate was added at 4°C, with rapid stirring of the solution, to the necessary degree of saturation. Unless otherwise stated 0.5–1h elapsed before the precipitate was separated by centrifugation at 26000*g* for 15min.

Tissue-space experiments. About 2g of sheep nasal septum cartilage was sliced into 1mm cubes and incubated at 37°C in 10ml of Krebs improved Ringer 1 medium, which had been saturated with CO_2, (Krebs, 1950) and which contained 1mM-glutamine and 5% (w/v) bovine serum albumin to maintain the osmotic pressure; the buffer was aerated with O_2+CO_2 (95:5) for 20min. The cubes were then transferred to a second container that was kept at 37°C and were incubated with the same volume of Ringer solution containing approximately 1×10^6 c.p.m. of $^{3}H_2O$ and 1×10^5 c.p.m. of [U-^{14}C]sucrose. Samples of cartilage (approx. 250mg) were removed at various times, blotted dry, quickly weighed and homogenized in 3 vol. of 0.75M-$HClO_4$ and left for 12h at 4°C. At zero time 0.1ml of the incubation mixture was taken and treated as above. The homogenates were neutralized with 6M-KOH, then they were centrifuged at 5000*g* for 10min and the supernatants were counted for ^{14}C and ^{3}H radioactivity.

Dry weight determination. Fresh cartilage (1.246g) was dried over silica gel at 100°C for 20h and the residue was weighed. The residue was dried for a further 20h and reweighed.

Purification of sheep nasal septum UDP-glucose dehydrogenase. Unless otherwise stated all steps were done at 4°C. After removal of the perichondrium 20–40g of fresh sheep (6–8 months old) nasal septum was sliced into thin slivers. The cartilage was then homogenized with 180ml (6vol.) of acetone at −20°C for 5min at full power (240V). The container was cooled intermittently with acetone–solid CO_2 mixture. The resulting viscous white homogenate was centrifuged at 11500*g* for 10min, and the supernatant was discarded. The residue was air-dried to remove excess of acetone. The dried residue was then ground with 0.3 of its own weight of sand in a precooled mortar until a fine powder was obtained, which was then homogenized with 200ml (7vol.) of 0.1M-sodium phosphate buffer, pH7.0, for 1.5min at full power. The homogenate was centrifuged at 26000*g* for 15min. The precipitate was discarded and ammonium sulphate (209g/l) was added to the supernatant to 35% saturation. The precipitate was removed and ammonium sulphate (474g/l) was added to 70% saturation. The precipitate was collected by centrifugation and resuspended in 3–5ml of 0.1M-sodium phosphate buffer, pH7.0, which contained 2mM-mercaptoethanol, and was then dialysed overnight against 4 litres of the same buffer. The precipitate formed on dialysis was spun down and the supernatant was used for kinetic studies.

Extraction of bovine corneal epithelial and stromal UDP-glucose dehydrogenase. The bovine eyes (140) were excised and placed on ice. The cornea was removed, and the epithelial layer was separated from the stromal and endothelial layers by scraping with a sharp scalpel. The epithelial scrapings were stored at −20°C until required. The endothelium was also removed by scraping, leaving the stroma intact.

Stromal tissue (62g) was homogenized with 300ml of 0.1M-sodium phosphate buffer–5mM-mercaptoethanol–4mM-EDTA, pH6.9, for 0.5min at low power (120V) and 0.5min at high power (240V). The viscous homogenate was centrifuged at 26000*g* for 15min and the supernatant containing small amounts of activity was discarded. The residue was re-homogenized with 300ml of −20°C acetone at full power for 2min and the residue after centrifugation at 15000*g* for 15min at −5°C was air-dried at 4°C. The dried powder was homogenized with 100ml of

0.02M-sodium phosphate buffer – 5mM-mercaptoethanol–2mM-EDTA, pH6.9, for 1min at full power (240V). The supernatant obtained after centrifugation at 15000*g* for 15min was brought to 35% saturation with solid ammonium sulphate (209g/l). The supernatant left after centrifugation was brought to 70% saturation with ammonium sulphate. The precipitate was collected and resuspended in 5ml of 0.02M-sodium phosphate buffer–5mM-mercaptoethanol–2mM-EDTA, pH6.9, and then was dialysed overnight against 2 litres of the same buffer.

Frozen corneal epithelium (8.5g) was powdered in a pre-cooled percussion mortar. The resulting powder was homogenized with 35ml of 0.02M-sodium phosphate buffer – 5mM-mercaptoethanol – 2mM-EDTA, pH6.9, for 0.5min at low power, followed by 0.5min full power. The homogenate was centrifuged at 15000*g* for 15min and the residue was discarded. The crude supernatant contained high NADH oxidase activity. Ammonium sulphate (209g/l) was added to 35% saturation. The precipitate was removed by centrifugation and solid ammonium sulphate (129g/l) was added to bring the supernatant to 55% saturation. The precipitate was collected and resuspended in 3ml of 0.01M-sodium phosphate buffer–5mM-mercaptoethanol–2mM-EDTA, pH6.9, and was dialysed overnight against 4 litres of the same buffer. The precipitate that formed on dialysis was spun down and the supernatant, which was devoid of NADH oxidase activity, was used for kinetic studies.

Extraction of neonatal rat skin UDP-glucose dehydrogenase. The skins from 2-day-old neonatal rats (Wistar strain) were collected and any excess fat was removed. The skins were immersed in 0.9% NaCl to remove excess of blood. The skins were stored at –20°C until required. Frozen skin (30g) was powdered in a percussion mortar and the powder was homogenized with 60ml of 0.05M-sodium phosphate buffer – 10mM-mercaptoethanol – 2mM-EDTA, pH6.9. The homogenate was centrifuged at 15000*g* for 15min and the residue was discarded. Solid ammonium sulphate was added to bring the supernatant to 35% saturation. The precipitate after centrifugation was discarded and the supernatant was brought to 55% saturation with ammonium sulphate. The precipitate was collected by centrifugation, and resuspended in 5ml of 0.02M-sodium phosphate buffer–10mM-mercaptoethanol–2mM-EDTA, pH6.9, and dialysed overnight against 2 litres of the same buffer.

Determination of sheep nasal cartilage sugar nucleotide concentrations. Nasal septum cartilage (11.045g) was deep-frozen in liquid N_2 and powdered in a pre-cooled percussion mortar. The powder was homogenized with 3vol. (30ml) of 0.75M-$HClO_4$ for 0.5min at full power and was clarified by centrifugation at 11500*g* for 10min. The residue was re-extracted twice more with 3vol. of 0.75M-$HClO_4$ and the supernatants were combined. They were left for 30min, adjusted to pH6.8 with saturated KOH, and left for a further 1h with readjustment of pH. The insoluble potassium perchlorate was spun down and the supernatant was applied to a column (15cm × 1cm) of Dowex-1 (formate form), prepared as described by Hurlbert *et al.* (1954). After adsorption of the contents of the supernatant on the column, the column was washed with water until a constant E_{254} was obtained. The column was next washed with 4M-formic acid, again until a constant baseline was obtained. Finally the column was washed with 4M-formic acid–0.4M-ammonium formate and the eluate fractions absorbing at 254nm were collected and combined. The combined fractions were desalted by slow passage through a Zeo-Karb 225 column (15cm × 1cm) followed by 2 bed volumes of water. The combined eluants were freeze-dried and resuspended in 0.2ml of water. Arabinitol (25nmol) and mannitol (100nmol) were added as internal standards. The samples were then prepared for analysis by g.l.c. on an F & M Biomedical Gas Chromatogram model 400 by the method of Bhatti *et al.* (1970).

Kinetic analysis: assay. The dehydrogenase activity was assayed in a 1ml capacity, 1cm-path-length cuvette containing 100μmol of sodium glycine buffer, pH8.7, 1μmol of NAD^+ and various amounts of the enzyme in a final volume of 1ml. The reaction was started by the addition of 1μmol of UDP-glucose and the increase in E_{340} due to NADH production was measured in an SP.1800 double-beam spectrophotometer with the appropriate blank. The temperature was maintained at 30°C and the reaction mixture was brought to this temperature by pre-incubation for 2.5min before the addition of the UDP-glucose. Initial rates were registered on a recorder by using a full-scale excursion of 0.1 extinction unit. Concentrations of substrates greater than 10K_m were assumed to saturate the enzyme.

In the inhibition experiments with UDP-xylose the variable substrate was preincubated with UDP-xylose and the enzyme, and the reaction was started by the addition of the saturating substrate.

A unit of enzyme is defined as the amount of enzyme required to produce 2μmol of NADH/min at 30°C.

Molecular-weight estimation with Sephadex G-200. A column (45cm × 1.5cm) of Sephadex G-200 was equilibrated with 20mM-sodium phosphate buffer–10mM-mercaptoethanol–2mM-EDTA, pH6.9, at 4°C. A mixture of 1ml of the dialysed enzyme from skin, together with 2mg of bovine catalase, 2mg of bovine serum albumin and 1.5mg of myoglobin was applied to the top of the column. The column was eluted at 3ml/h with the equilibration buffer and 0.5ml fractions were collected. Catalase and myoglobin were determined by measuring their E_{404}

and serum albumin was measured at 280nm. A plot of the logarithm of the molecular weight versus elution volume was used to calculate the molecular weight of the skin UDP-glucose dehydrogenase. The same procedure was used to determine the molecular weight of the sheep nasal septum enzyme.

Activation energy of sheep nasal septum UDP-glucose dehydrogenase. Nasal septum enzyme was incubated for 7min at the selected temperatures with 1μmol of NAD^+ and 100μmol of sodium glycine in a final volume of 1ml. The reaction was started by the addition of 1μmol of UDP-glucose and the initial velocity was measured. Experiments were done in duplicate.

The activation energy of the reaction was calculated from the slope of a plot of log k (rate constant) versus 1/°K.

Effect of pH on UDP-xylose inhibition of sheep nasal septum UDP-glucose dehydrogenase. The initial rate of production of NADH in 0.1M-tris–HCl buffer at various pH values was measured at 340nm at 35°C at physiological concentrations of UDP-glucose and NAD^+ (0.1mM and 1mM respectively), with and without physiological concentrations of UDP-xylose (0.01mM). The above procedure was repeated with 0.1M-sodium glycine buffer between pH8.0 and 9.1. The results were presented as a plot of the logarithm of percentage inhibition against pH.

UDP-glucuronic acid nucleotidase activity in sheep nasal septum. Nasal septum (6.7g from 6–8 month-old sheep) was cut into small cubes, frozen in liquid N_2 and powdered in a pre-cooled percussion mortar. The powder was ground with 40ml of 0.154M-KCl and 10g of purified sand. The homogenate was squeezed through cheese-cloth and the filtrate was centrifuged at 10000g for 30min. The supernatant was decanted and dialysed overnight against 4 litres of water. The resulting dialysis residue was centrifuged at 10000g for 15min and the precipitate was removed. A sample (0.5ml) of the supernatant was incubated with shaking at 37°C with 3.33×10^5c.p.m. of UDP-D-[U-^{14}C]glucuronic acid, 0.16mg of carrier UDP-glucuronic acid and 0.5ml of 50mM-sodium phosphate buffer, pH 7.4. A control containing 0.5ml of enzyme solution that had been heated at 100°C in a boiling-water bath for 2min was also used. A third incubation mixture containing enzyme, UDP-glucuronic acid and 10mM-EDTA was also used.

The course of the reaction in the various incubations was followed by removing 0.1ml samples at 30, 80, 200 and 300min. The samples were heated at 100°C for 2min, then 0.6ml of 50mM-sodium phosphate buffer, pH7.4, was added followed by 6mg of Norit A. The mixture was left for 5min and then filtered by suction through a disc of Whatman GF/B glass paper. The Norit A residue was washed with a further 2ml of water. Fractions (0.2ml) of the various filtrates were removed, mixed with 10ml of scintillator and counted for ^{14}C radioactivity.

Identification of glucuronic acid 1-phosphate. Nasal septum extract (1ml) containing nucleotidase activity was incubated with 6×10^5c.p.m. of UDP-[U-^{14}C]glucuronic acid and 0.48mg of carrier UDP-glucuronic acid in a final volume of 2ml of 50mM-sodium phosphate buffer, pH7.4. After 5h at 37°C the incubation was heated at 100°C for 2min and the protein precipitate was removed by centrifugation. Norit A (20mg) was added to the supernatant and after 5min the whole was filtered, with suction, through glass-paper discs. The filtrate was adsorbed on a column (2cm×1cm) of Dowex-1 (formate form) and the column was eluted with 4 bed volumes of 4M-formic acid–0.1M-ammonium formate mixture and the eluate was discarded. The column was next eluted with 4M-formic acid–0.8M-ammonium formate. The eluates were collected and passed slowly through a column (4.5cm×1cm) of Zeo-Karb 225 (H^+ form), followed by 2 bed volumes of water.

The freeze-dried eluate was then resuspended in 20μl of water and 0.1mg of glucuronic acid 1-phosphate was added. The mixture was then chromatographed on Whatman 3MM paper. UDP-glucuronic acid and glucuronic acid 1-phosphate were run as standards. The chromatogram was dried at 60°C for 5min and UDP-glucuronic acid was identified by using u.v. light at 254nm. Glucuronic acid 1-phosphate was identified by the method of Burrows *et al.* (1952) for organic and inorganic phosphate groups. The chromatogram was also scanned for radioactivity by using a Tracerlab 4π radioactivity scanner (Tracerlab, Waltham, Mass., U.S.A.).

Steady-state concentrations of glucuronic acid 1-phosphate in sheep nasal cartilage. Frozen nasal cartilage (8g) was powdered in a percussion mortar. The powder was homogenized for 1min with 16ml of 0.75M-$HClO_4$. The homogenate was centrifuged at 15000g for 15min and the residue was collected and rehomogenized with 10ml of 0.75M-$HClO_4$. After 0.5h at 4°C the combined supernatants were adjusted to pH6.8 by the addition of concentrated KOH and left for a further 1h with re-adjustment of pH if necessary. The insoluble $KClO_4$ was removed by centrifugation at 10000g for 15min. The supernatant was adsorbed on a column (4cm×0.5cm) of Dowex-1 (formate form). The column was washed with 3 bed volumes of 4M-formic acid–0.2M-ammonium formate and the eluate was discarded. The column was then washed with 3 bed volumes of 4M-formic acid–0.8M-ammonium formate and the resulting eluates were passed slowly through a column (6cm×1cm) of Zeo-Karb 225 (H^+ form) followed by 2 bed volumes of water. The freeze-dried eluate was resuspended in 10μl of water and chromatographed on Whatman 3MM paper,

together with UDP-glucuronic acid and glucuronic acid 1-phosphate standards. The area corresponding to the glucuronic acid 1-phosphate was cut out and further eluted with solvent. The eluate was passed through Zeo-Karb 225 (H^+ form) followed by 2 bed volumes of water and the eluates were collected and freeze-dried. The freeze-dried material was resuspended in 0.1ml of water, then 25nmol each of arabinitol and mannitol were added as internal standards and the samples were prepared for g.l.c. by the method of Bhatti *et al.* (1970).

UDP-glucuronic acid pyrophosphorylase activity in nasal septum cartilage. Sheep nasal cartilage not containing the vascular part of the septum was frozen and powdered in a percussion mortar. The powder was ground in a pre-cooled mortar with 10ml of 60mM-tris–HCl–5mM-mercaptoethanol buffer, pH7.4. The paste was centrifuged at 10000*g* for 15min, and the supernatant was decanted. A sample (0.5ml) of supernatant was incubated at 37°C with 10000c.p.m. of [U-^{14}C]glucuronic acid 1-phosphate (prepared as above from UDP-[U-^{14}C]glucuronic acid and UDP-glucuronic acid nucleotidase), 2μmol of mercaptoethanol, 5μmol of UTP, 2μmol of $MgCl_2$, 6μg of crystalline inorganic pyrophosphatase, 30μmol of tris–HCl buffer, pH7.4, all in a final volume of 1ml. After 2h, the incubation mixture was heated at 100°C for 2min, then the precipitate was removed by centrifugation, and 12mg of Norit A was added to the supernatant. After 5min the mixture was filtered with suction through glass-fibre discs. The Norit A residue remaining on the filter was washed with 7ml of water. The Norit was removed from the glass-fibre disc and agitated with 3ml of aq. 50% ethanol–0.15M-ammonia. The mixture was filtered as above and the filtrate was freeze-dried. The freeze-dried material was resuspended in a small volume of water, mixed with 10ml of scintillator and counted for ^{14}C radioactivity.

Maximum and physiological rates of UDP-glucuronic acid synthesis in various tissues. The maximum rate of UDP-glucuronic acid biosynthesis was established at pH8.7 and 36°C by using 100μmol of sodium glycine buffer, 1μmol of NAD^+ and 1μmol of UDP-glucose in a final volume of 1ml. The initial velocities of NADH production were measured at 340nm.

Physiological rates were established as above by using the various physiological concentrations of UDP-glucose and UDP-xylose found for each tissue; 1μmol of NAD^+ was used, assuming the intracellular concentration of NAD^+ to be saturating.

Rates of synthesis were expressed in μmol/min per g wet wt. of tissue.

Results

pH optimum

Nasal septum cartilage UDP-glucose dehydrogenase gave a sharp pH profile in 0.1M-sodium glycine buffer with an optimum at pH8.7 at 30°C.

Effect of various substrate concentrations

The effect of various NAD^+ concentrations at a constant saturating UDP-glucose concentration for the nasal septum and neonatal skin enzymes is shown in Fig. 1(*a*). The apparent K_m values at 30°C as determined graphically were 1.25×10^{-4} and 1.54×10^{-4}M respectively.

The effect of various UDP-glucose concentrations at a saturating NAD^+ concentration for the nasal septum, neonatal skin and corneal epithelial enzymes is shown in Fig. 1(*b*). The K_m values were 5.5×10^{-5}, 4.3×10^{-5} and 3.3×10^{-5}M respectively.

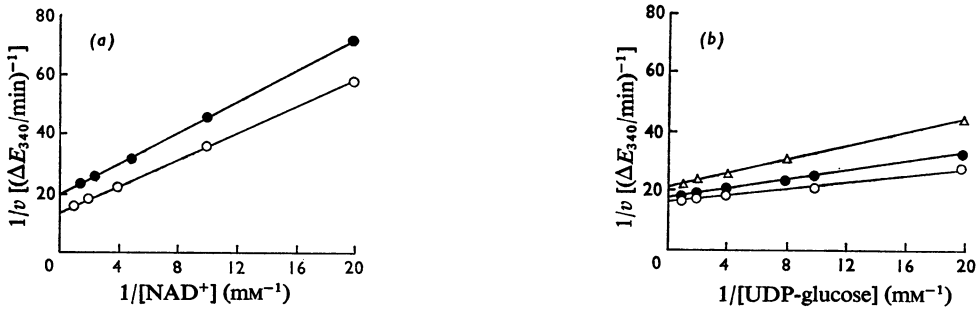

Fig. 1. *Reciprocal plots of velocity versus substrate concentration*

(*a*) NAD^+ as the variable substrate with saturating UDP-glucose (1mM): o, neonatal rat skin; ●, sheep nasal septum. (*b*) UDP-glucose as the variable substrate with saturating NAD^+ (1mM): o, bovine corneal epithelium; ●, neonatal rat skin; △, sheep nasal cartilage.

Molecular weight

A linear relationship was found on plotting the logarithm of the molecular weight of the protein standards versus elution volume. Both skin and nasal septum enzymes were eluted just before the catalase marker, indicating a molecular weight in excess of 260000. Extrapolation of the UDP-glucose dehydrogenase elution volume, on the linear logarithm of molecular weight versus elution volume plot, indicated a molecular weight of about 3×10^5 for both enzymes, assuming both of the dehydrogenases to be approximately spherical.

UDP-xylose inhibition

UDP-xylose acts as a potent inhibitor of the UDP-glucose dehydrogenase from all three sources with respect to both substrates, UDP-glucose and NAD^+. Reciprocal plots of reaction velocity versus UDP-glucose or NAD^+ concentration at various UDP-xylose concentrations yielded a family of curves that showed an upward curvature especially at low concentrations of UDP-glucose or NAD^+ and high concentrations of UDP-xylose (Figs. 2 and 3).

At low inhibitor concentrations the inhibition with respect to UDP-glucose and NAD^+ for the neonatal skin enzyme appears to be of a competitive nature, as shown by the constant $V_{max.}$.

At higher concentrations of inhibitor however, the inhibition becomes more complex. The inhibition with respect to UDP-glucose in corneal epithelium also appears to be competitive at low inhibitor concentrations. The inhibition of sheep nasal septum enzyme appeared to be competitive with respect to NAD^+ and UDP-glucose; however, the kinetic parameters with respect to the latter were not as clear as those for the skin and corneal epithelial enzymes, as shown in Fig. 2 where the $V_{max.}$ intercept appears dependent on the concentration of UDP-xylose.

The results for UDP-xylose inhibition with respect to UDP-glucose were replotted as log $[(V-v)/v]$ versus log (inhibitor concentration) at various UDP-glucose concentrations. In each case a family of parallel lines was obtained with n values between 1.4 and 1.6, and is indicative of co-operation between the sub-units of this enzyme (for discussion see Taketa & Pogell, 1965). Fig. 4 shows such a plot for neonatal rat skin enzyme.

Gas–liquid chromatography of sugars

The g.l.c. elution profile of the neutralized $HClO_4$ extract from sheep nasal septum cartilage is shown in Fig. 5. The concentrations of the individual sugars are given in Table 1. It is assumed that sugars were originally present as the UDP derivatives unless otherwise stated, because of their position of elution

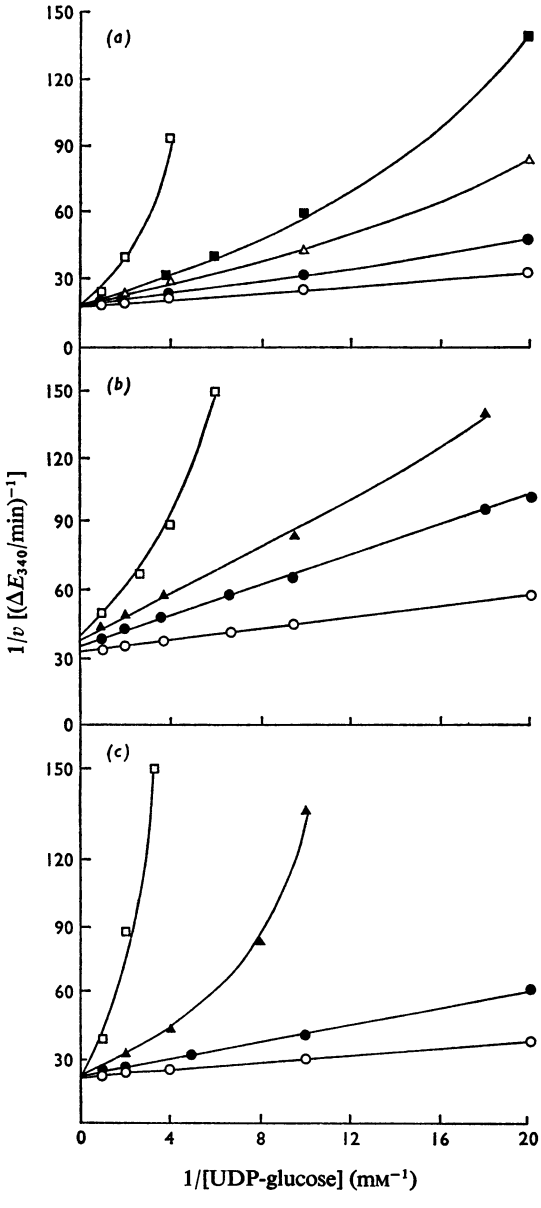

Fig. 2. *Reciprocal plots of velocity versus UDP-glucose concentration at constant saturating NAD^+ concentrations and various concentrations of UDP-xylose for UDP-glucose dehydrogenase from various sources*

(*a*) Neonatal rat skin enzyme: o, nil; ●, 10μM; △, 20μM; ■, 25μM; □, 50μM-UDP-xylose. (*b*) Sheep nasal cartilage enzyme: o, nil; ●, 5μM; ▲, 10μM; □, 25μM-UDP-xylose. (*c*) Bovine corneal epithelium enzyme: o, nil; ●, 5μM; ▲, 20μM; □, 40μM-UDP-xylose.

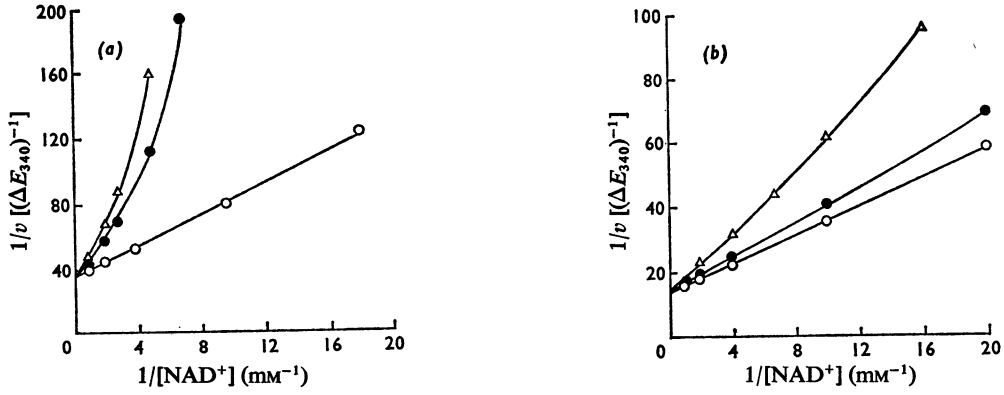

Fig. 3. *Reciprocal plots of velocity versus NAD$^+$ concentration at constant saturating UDP-glucose concentrations and various concentrations of UDP-xylose for UDP-glucose dehydrogenase from various sources*

(*a*) Sheep nasal cartilage enzyme: o, nil; ●, 5μM; △, 10μM-UDP-xylose. (*b*) Neonatal rat skin enzyme: o, nil; ●, 5μM; △, 10μM-UDP-xylose.

from Dowex-1 (formate form). UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine were also present; however, an estimation of their concentrations was not attempted because of the variable base-line in this region of the chromatogram.

Mannose and fucose, presumably as their GDP derivatives were also present, but no estimations of their concentrations were made.

Tissue-space experiment

The difference between the total [^{14}C]sucrose-penetrable space and the total ^3H_2O-permeable space gives a value for the cell water volume. For sheep nasal septum cartilage, the sucrose-penetratable space was 54% of the wet weight of tissue, whereas that for ^3H_2O was 68% of the wet weight. Hence by difference the cell water is equivalent to 14% of the wet weight.

The difference between the total water space (76%), as determined by oven drying, and the ^3H_2O-permeable space (68%), gives an indication of the bound or unavailable water content of cartilage, and is about 8%.

The cellular water content was used to calculate the cellular concentrations of metabolites involved in glycosaminoglycan biosynthesis.

Activation energy

As shown in Fig. 6 a linear relationship is obtained on plotting log *k* (rate constant) versus the reciprocal of the absolute temperature. The activation energy as determined from the slope is 40kJ/mol.

Effect of pH on UDP-xylose inhibition

Fig. 7 shows a plot of log (percentage inhibition) versus pH obtained by using either sodium glycine or tris–HCl buffers which yielded a sigmoid-shaped curve, with a p*K* value, at the inflexion point, of 8.3–8.4. Fig. 7 also shows that, whereas the percentage inhibition increased rapidly with decrease in pH, the enzymic activity decreased very little over the same pH range.

UDP-glucuronic acid nucleotidase activity

When UDP-glucuronic acid was incubated with an extract of sheep nasal septum, the increase in radioactivity of glucuronic acid 1-phosphate with time shown in Fig. 8 indicates the presence of UDP-glucuronic acid nucleotidase activity. The nucleotidase activity was inhibited 92% by 10mM-EDTA. The rate of synthesis of glucuronic acid 1-phosphate was calculated to be about 2nmol/min per g wet wt. The product of the reaction was identified as glucuronic acid 1-phosphate by comparison with the chromatographic behaviour of authentic glucuronic acid 1-phosphate previously added to the incubation mixture. Separation was achieved by passage through Dowex-1 (formate form) and Zeo-Karb 225 (H^+ form).

Steady-state concentrations of glucuronic acid 1-phosphate

The g.l.c. traces of the neutralized $HClO_4$ extract of sheep nasal septum showed glucuronic acid was not present. Thus the steady-state concentration of

glucuronic acid 1-phosphate seems to be very low, if not negligible. This point is discussed below.

UDP-glucuronic acid pyrophosphorylase activity

To determine if glucuronic acid 1-phosphate could be recycled to UDP-glucuronic acid by a pyrophosphorylase activity, an extract of sheep nasal septum cartilage in 30mM-tris–HCl was incubated with UTP, Mg^{2+} and [U-^{14}C]glucuronic acid 1-phosphate. After 2h, separation of the products on Norit A, and examination of the aq. 50% ethanol–0.15M-ammonia wash showed no radioactive UDP-glucuronic acid. Hence it is assumed from this experiment that the relevant pyrophosphorylase is absent.

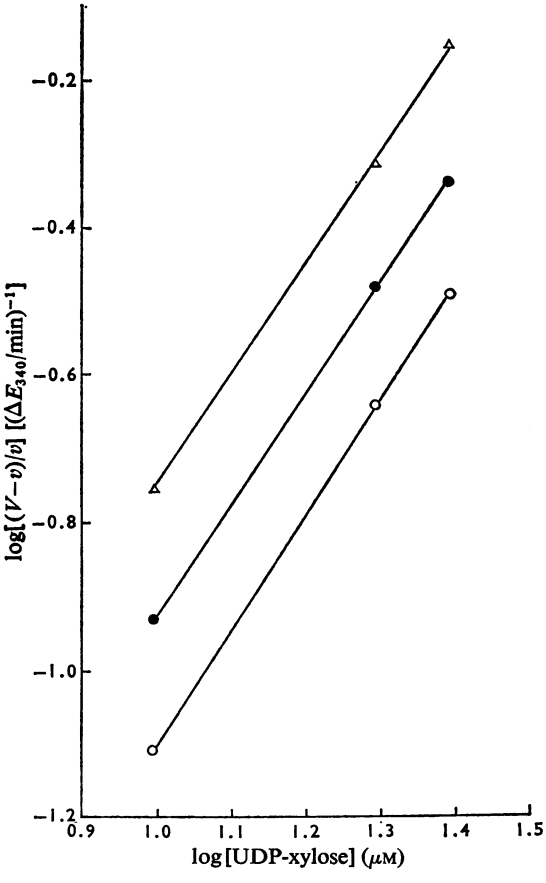

Fig. 4. *Plot of log [(V−v)/v] versus log (UDP-xylose concentration) at various UDP-glucose concentrations and constant saturating NAD$^+$ concentration for neonatal rat skin UDP-glucose dehydrogenase*

○, 0.5mM; ●, 0.25mM; △, 0.167mM-UDP-glucose.

Maximum and physiological rates of UDP-glucuronic acid biosynthesis

The maximum rates at pH8.7 of UDP-glucuronic acid biosynthesis in nasal septum, skin and corneal epithelial tissues are about 20, 7.7 and 10nmol/min per g wet wt. respectively. Physiological rates were calculated to be 11 and 5nmol/min per g wet wt. for nasal septum and skin respectively at pH8.7. The physiological rate of UDP-glucuronic acid synthesis in corneal epithelium was measured at a UDP-xylose concentration (40μM) that was half of the physiological value because of difficulties in measuring very low rates of NADH synthesis at 80μM-UDP-xylose. Assuming a linear relationship between percentage inhibition and UDP-xylose concentration, the rate of synthesis would be less than 0.4nmol/min per g wet wt. The rate of synthesis of UDP-glucuronic acid in corneal stroma was not measured because of the small amounts of enzyme obtained from this tissue.

Studies with tris–HCl buffer indicate that the rate of production of UDP-glucuronic acid at pH7.5 is about 0.3 of that at pH8.7. Assuming the intracellular pH to be about 7.5 the above physiological rates would be further decreased to 3.7, 1.7 and 0.1nmol/min per g wet wt. respectively.

Discussion

The properties of UDP-glucose dehydrogenase from sheep nasal septum cartilage, neonatal rat skin and bovine corneal epithelium are very similar to those shown for the enzyme from other sources (Table 2).

The response of each enzyme to UDP-xylose is similar to that reported for the enzyme from pea and liver (Neufeld & Hall, 1965), yeast (Ankel *et al.*, 1966), hen oviduct (Bdolah & Feingold, 1968) and chick embryo liver and eye (Darrow & Hendrickson, 1971). The inhibition with respect to both substrates is of a complex nature, as shown by the upward curvature of the $1/v$ versus $1/s$ plots at low UDP-glucose and NAD$^+$ concentrations. The inhibition appears to be of an allosteric nature, as indicated by the 'n' values of 1.4–1.6 for each enzyme from the various sources, and the sigmoid, rather than Michaelis–Menten type, kinetics obtained on plotting the logarithm of percentage inhibition versus inhibitor concentration. UDP-xylose inhibition is no longer apparent at high concentrations of either substrate in each case.

G.l.c. has shown the concentrations of UDP-glucose and UDP-xylose in nasal cartilage to be about 73μM and 7μM respectively. The nasal cartilage enzyme is about 42% inhibited at physiological UDP-xylose, UDP-glucose and NAD$^+$ concentrations (Fig. 2*b*).

Assuming the cell water content of neonatal rat skin to be 35% of wet weight (Hardingham & Phelps, 1968) then the intracellular concentrations of UDP-

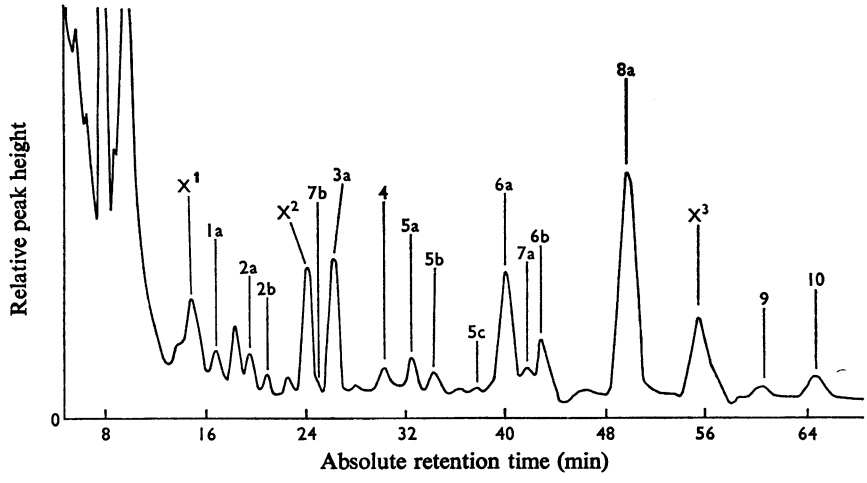

Fig. 5. *Separation of the monosaccharides of sheep nasal septum cartilage by g.l.c.*

The monosaccharides were separated as the trimethylsilyl esters of the *O*-methyl glycosides as described by Bhatti *et al.* (1970). The main peaks are listed below. 1a, Fucose; 2a,b, xylose; 3a, arabinitol; 4, mannose; 5a,b,c, galactose; 6a,b, glucose; 7a,b, glucuronic acid; 8a, mannitol; 9, *N*-acetylgalactosamine; 10, *N*-acetylglucosamine. X^1, X^2 and X^3 represent unidentified peaks that are present in a number of biological samples (J. R. Clamp, personal communication).

Table 1. *Sugar nucleotide content of sheep nasal septum cartilage*

For experimental details see the text. Values are given as means ± S.D. of three experiments.

Sugar nucleotide	Total (nmol/11.045g)	Cellular concentration (μM)
UDP-xylose	11.0±0.365	7.0
UDP-glucose	113.0±2.58	73.0
UDP-galactose	37.0±0.12	24.0
UDP-glucuronic acid	43.0±5.56	28.0

xylose (C. J. Handley & C. F. Phelps, unpublished work) and UDP-glucose (Hardingham & Phelps, 1968) are 10μM and 210μM respectively. The skin enzyme is 20–25% inhibited at physiological concentrations of substrates and inhibitor (Fig. 2*a*).

The UDP-glucose concentrations in bovine corneal epithelium and stroma are 279μM and 364μM respectively (Handley & Phelps, 1972*b*). The UDP-xylose concentrations are 85μM and 72μM respectively. The corneal epithelial enzyme is more than 95% inhibited (Fig. 2*c*). Inhibition studies with the enzyme from corneal stroma with physiological concentrations of substrates and inhibitor have shown it to be similarly inhibited.

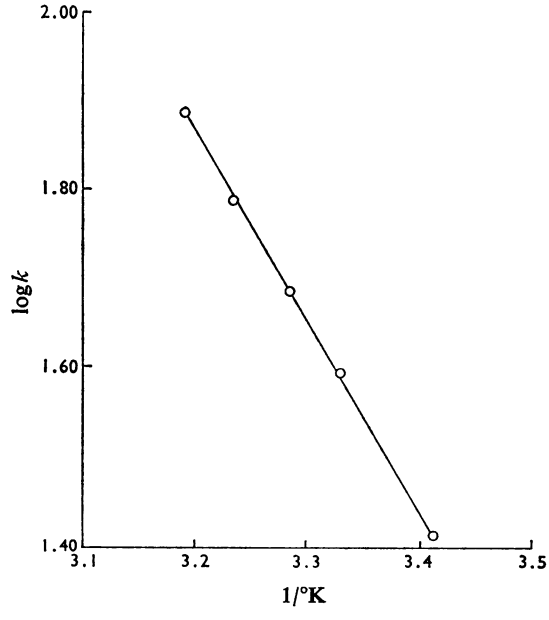

Fig. 6. *Effect of temperature on the reaction rate of sheep nasal septum cartilage UDP-glucose dehydrogenase*

Both NAD^+ and UDP-glucose concentrations were saturating. For further details see the text.

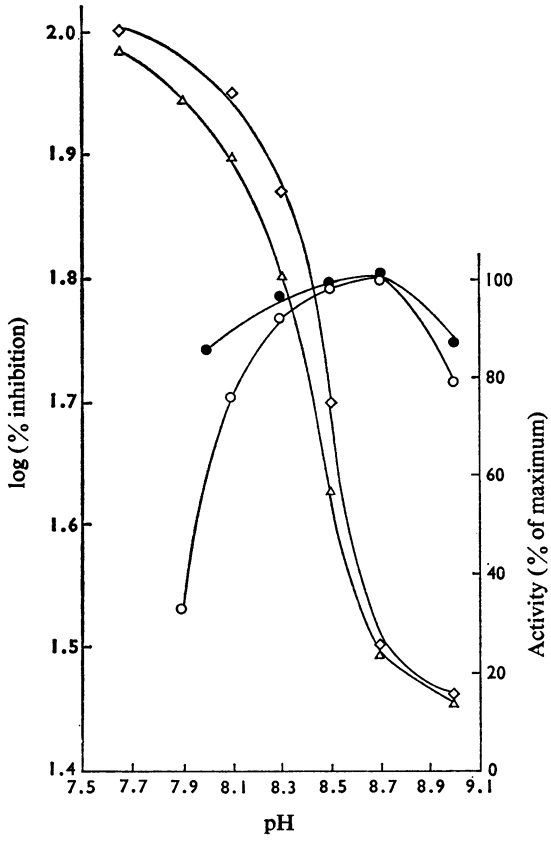

Fig. 7. *Effect of pH on the inhibition by UDP-xylose of sheep nasal septum cartilage UDP-glucose dehydrogenase*

Inhibition with 0.1 M-sodium glycine buffer, △; with 0.1 M-tris–HCl buffer, ◇. Uninhibited rates at various pH values with 0.1 M-tris–HCl buffer, ●; with 0.1 M-sodium glycine buffer, ○.

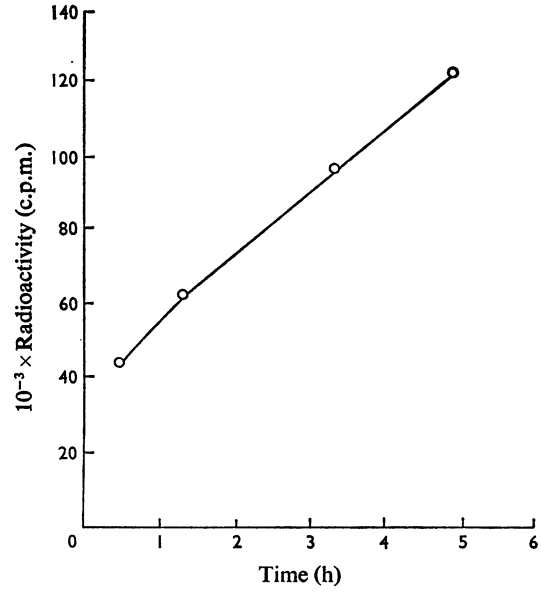

Fig. 8. *Rate of production of [U-^{14}C]glucuronic acid 1-phosphate from UDP-[U-^{14}C]glucuronic acid by sheep nasal cartilage UDP-glucuronic acid nucleotidase*

For experimental details see the text. Each observation represents the mean of two experiments.

These interpretations of the results assume that there is no compartmentation of enzyme, substrates or inhibitor within the intracellular space. It is also assumed that the NAD^+ concentration is nearly saturating at about 1 mM. If the NAD^+ concentration is lower than this, the inhibitory power of UDP-xylose will be increased.

The degree of inhibition observed in the various tissues at physiological concentrations of substrates and inhibitor is consistent with the types of proteoglycan produced by each of the tissues. In nasal septum cartilage the major proteoglycan is chondroitin sulphate (Hascall & Sajdera, 1970), which contains glucuronic acid as a component of its disaccharide repeating unit. Thus the level of inhibition imposed on UDP-glucose dehydrogenase by UDP-xylose is slight.

The major proteoglycans of neonatal rat skin are hyaluronic acid (56%), dermatan sulphate (16%) and chondroitin sulphate (9%) (Hardingham & Phelps, 1970). Both hyaluronic acid and chondroitin sulphate contain glucuronic acid, whereas dermatan sulphate contains L-iduronic acid (formed by C-5 epimerization of glucuronic acid) as components of their disaccharide repeating unit. These observations are consistent with the fact that in neonatal rat skin the UDP-glucose dehydrogenase appears to be 20–25% inhibited under steady-state conditions. Handley & Phelps (1972*e*) have shown that keratan sulphate represents 71% of the total protein–carbohydrate-linked material in bovine corneal stroma. The component sugars of the disaccharide repeating unit of keratan sulphate are *N*-acetylglucosamine and galactose. Chondroitin sulphate (21%) is a minor component of this tissue. Thus the comparatively low amounts of glucuronic acid-containing polymers in this tissue may be a reflexion of the high degree of inhibition exerted on the UDP-glucose dehydrogenase by UDP-xylose.

The degree of inhibition assumed in bovine corneal

Table 2. *Comparison of UDP-glucose dehydrogenase from various sources*

Source	K_m NAD$^+$ ($10^4 \times$ M)	K_m UDP-glucose ($10^5 \times$ M)	pH optimum	Molecular weight	References
Pea seedling	1.15	7.0	9.0	—	Strominger & Mapson (1957)
Rat liver	1.2	1.2	8.7	300000	P. A. Gainey & C. F. Phelps (unpublished work)
Yeast	6.0	60.0	7.3–7.8	—	Ankel *et al.* (1966)
Rabbit skin	—	2.4	9.0	—	Jacobson & Davidson (1962)
Hen oviduct	0.9	4.0	9.0	⩾100000	Bdolah & Feingold (1968)
Nasal cartilage	1.25	5.5	8.7	⩾260000	Present work
Neonatal rat skin	1.54	4.3	—	⩾260000	Present work
Bovine corneal epithelium	—	3.3	—	—	Present work

epithelium is not as yet completely understood. Little information is available about the synthesis of proteoglycans in this tissue, although Robert & Robert (1970) have investigated the glycoprotein content of this tissue.

An explanation for the presence in corneal epithelium of such high concentrations of UDP-xylose, which are presumably formed by decarboxylation of UDP-glucuronic acid, is also not immediately obvious.

The inhibition observed with both the corneal stroma and epithelium enzymes is consistent with the work of Balduini *et al.* (1970), who have shown that the addition of UDP-xylose to cornea *in vitro* resulted in a significant decrease in chondroitin sulphate synthesis. Work on the sugar nucleotide pool size of neonatal rat epiphyses (Handley & Phelps, 1972*a*) has also suggested regulation at the UDP-glucose dehydrogenase level.

The apparent increase in UDP-xylose inhibition of nasal cartilage UDP-glucose dehydrogenase with decrease in pH is shown in Fig. 7. The uninhibited rate remains approximately constant over the same pH range. This observation is consistent with the existence of a UDP-xylose-binding site distinct from the active site(s). The pK value determined from the point of inflexion of the curve in Fig. 7 is 8.3–8.4.

The conversion of glucuronic acid 1-phosphate into UDP-glucuronic acid, utilizing a specific UDP-glucuronic acid pyrophosphorylase, has been demonstrated in several plant species (Feingold *et al.*, 1958; Marsh, 1960). Mammals do not seem to possess a similar pathway (Storey & Dutton, 1955; Smith & Mills, 1954). A single contrary report (Arias *et al.*, 1958) has not been confirmed. Jeffrey & Rienits (1970), using embryonic chick epiphysial cartilage, have, however, indicated that α-glucuronic acid 1-phosphate may react, presumably with UTP, to yield UDP-glucuronic acid. Work presented above does not support the idea that UDP-glucuronic acid pyrophosphorylase exists in mammalian tissues. UDP-glucose is therefore presumably the major precursor of UDP-glucuronic acid.

Studies utilizing UDP-[U-^{14}C]glucuronic acid have shown the presence of an active UDP-D-glucuronic acid nucleotidase that converts UDP-D-glucuronic acid into UMP and glucuronic acid 1-phosphate. Pogell & Krisman (1960), Pogell & Leloir (1961) and Conney & Burns (1961) have shown similar activity in rat skin, liver and kidney particulate fractions, respectively. The rate of synthesis of glucuronic acid 1-phosphate was estimated to be about 2nmol/min per g wet wt. of tissue. Handley & Phelps (1972*a*) have shown the rate of UDP-glucuronic acid synthesis in neonatal rat epiphysial cartilage to be 0.8nmol/min per g wet wt. Assuming a similar rate of UDP-glucuronic acid synthesis in nasal cartilage, it is apparent that the rate of utilization of UDP-glucuronic acid would exceed the rate of synthesis if it were being converted into glucuronic acid 1-phosphate. As g.l.c. has demonstrated UDP-glucuronic acid to be present at a significant concentration under steady-state conditions, and glucuronic acid 1-phosphate in negligible amounts, it would appear that *in vivo* the major source at least of glucuronic acid 1-phosphate is not UDP-glucuronic acid. Pogell & Krisman (1960) have reported the UDP-glucuronic acid nucleotidase from skin to be inhibited by UDP-*N*-acetylglucosamine, UDP-glucose and EDTA. Nasal septum nucleotidase was highly inhibited by EDTA. Intracellular concentrations of UDP-*N*-acetylglucosamine and UDP-glucose may act to inhibit the skin nucleotidase *in vivo*.

Alternatively the absence of significant concentrations of glucuronic acid 1-phosphate could be explained by assuming that a very active glucuronic acid 1-phosphate phosphatase is present. Such an enzyme has been identified in rat liver, kidney and

skin homogenates. Pogell & Krisman (1960) have suggested that the conversion of UDP-glucuronic acid into glucuronic acid 1-phosphate and subsequently into glucuronic acid are the first steps in the synthesis of ascorbic acid. However, this hypothesis remains to be tested.

The rate of UDP-glucuronic acid synthesis *in vitro* in neonatal rat skin and epiphysial cartilage is about 0.24 and 0.8nmol/min per g wet wt. of tissues respectively (Hardingham & Phelps, 1968; Handley & Phelps, 1972*a*). The apparent physiological rates observed *in vitro* at pH7.5 in the present work were 1.7 and 3.7nmol/min per g wet wt. respectively. The rates observed *in vitro* for the neonatal rat skin enzyme are in good agreement.

However, the increase in UDP-xylose inhibition with a decrease in pH would lead to an inhibition of the nasal septum UDP-glucose dehydrogenase in the presence of physiological concentrations of substrates and inhibitor greater than 95% at pH8.0. Further, this inhibition is overcome only by relatively large concentrations of either substrate compared with those concentrations required at pH8.7. Thus at pH values approaching those believed to exist intracellularly, there might be expected to be a negligible production of UDP-glucuronic acid. This is in contrast with the reported steady-state values for UDP-glucuronic acid production (Handley & Phelps, 1972*a*; Hardingham & Phelps, 1968). This apparent contradiction might be explained by assuming either that the enzyme is associated with membrane structures that are capable of modifying its kinetic parameters or the pH of its environment, or that there is compartmentation of the UDP-xylose between the cisternal space of the endoplasmic reticulum and the cytoplasm. UDP-glucose dehydrogenase is membrane-bound only in bacteria (N. Norval & I. W. Sutherland, unpublished work). Careful cellular fractionation studies performed in this laboratory with rat liver have shown the enzyme to be localized in the cytoplasm. It is unlikely that UDP-xylose is compartmented as this would indicate that the UDP-xylose does not act as a regulator of UDP-glucose dehydrogenase activity, when there is a reasonable amount of information available which suggests that it does.

Interestingly the concentrations of the various sugar nucleotides in bovine nasal septum cartilage are similar to those in neonatal rat skin epiphysial cartilage. The ratio of UDP-*N*-acetylglucosamine to UDP-*N*-acetylgalactosamine, and that of UDP-glucose to UDP-galactose, measured by g.l.c. are similar to the free equilibrium values reported by Handley & Phelps (1972*a*) for rat epiphysial cartilage. The UDP-glucuronic acid concentration in nasal cartilage was about 30μM. The UDP-glucuronic acid concentrations in neonatal rat epiphysial cartilage and skin are 80μM and 90μM respectively (Handley & Phelps, 1972*a*; Hardingham & Phelps, 1968). Handley & Phelps (1972*b*) have also shown the UDP-glucuronic acid concentration in corneal stroma to be about 60μM. Assuming that the UDP-glucose dehydrogenase from each of these sources has a K_i value for UDP-glucuronic acid (0.14mM) similar to that of the rat liver enzyme (P. A. Gainey & C. F. Phelps, unpublished work), it is apparent that the dehydrogenase from each source would be inhibited by UDP-glucuronic acid. Whether such inhibition is significant *in vivo* is not known.

The above findings relate to the normal steady-state conditions in the tissues described. It is to be expected that under abnormal conditions such as are experienced with certain drugs, hormones and states of nutrition that the values reported here may change. Bentley *et al.* (1971) have detailed the changes occurring in hyaluronic acid production in the sex skin of monkeys treated with oestrogen. Anti-rheumatic drugs such as cortisol and prednisolone have been shown to affect the rates of turnover of hyaluronate and chondroitin sulphates in rat skin (Schiller & Dorfman, 1957). Caygill (1968) has extensively reviewed the effects of drugs and hormones on glycosaminoglycan biosynthesis.

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