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The Biosynthesis *in vitro* of Keratan Sulphate in Bovine Cornea

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1. Bovine corneas were incubated *in vitro* with [U-^{14}C]glucose. 2. The glycosaminoglycans of corneal stroma were isolated and fractionated on cetylpyridinium chloride–cellulose columns. The major components were keratan sulphate (71%), chondroitin 4-sulphate (17%) and chondroitin 6-sulphate (4%). 3. The acid-soluble nucleotides and intermediates of glycosaminoglycan biosynthesis of corneal stroma were separated on Dowex 1 (formate form) and the tissue content and cellular concentrations were determined. 4. The rates of synthesis of the intermediates of glycosaminoglycan biosynthesis in corneal stroma were determined. 5. The incorporation of radioactivity from [U-^{14}C]glucose into the uronic acid and hexosamine components of the glycosaminoglycans present in corneal stroma were measured and the turnover rates of these polymers were calculated. It was found that keratan sulphate was turning over in about 723 h and chondroitin 6-sulphate in 251 h.

The biosynthesis of glycosaminoglycans in neonatal rat skin (Hardingham & Phelps, 1968, 1970*a*,*b*) and in neonatal rat epiphysial cartilage (Handley & Phelps, 1972) has been investigated by following the time-course of incorporation of [U-^{14}C]glucose through each of the intermediates of polymer synthesis into the acidic polysaccharides produced. Neonatal rat skin produces a wide spectrum of glycosaminoglycans, hyaluronic acid being predominant. In neonatal rat epiphysial cartilage the chondroitin sulphates make up over 80% of the total acidic polysaccharide content. Although these two tissues synthesize different glycosaminoglycans, hyaluronic acid being a glucosaminoglucuronan whereas chondroitin sulphate possesses a sulphated galactosaminoglucuronan repeating unit, it has been shown by Hardingham & Phelps (1968, 1970*b*) and by Handley & Phelps (1972) that the flux of radioisotopic carbon through the intermediates of these polymers in these two tissues is substantially the same.

The work described in this paper investigates the biosynthesis of keratan sulphate in bovine corneal stroma and was undertaken to explore in what way the synthesis of a non-glucuronic acid-containing polymer differs from glycosaminoglucuronan biosynthesis investigated in previous studies (Hardingham & Phelps, 1968, 1970*b*; Handley & Phelps, 1972).

Experimental

Materials were as described by Handley & Phelps (1972).

Methods

Centrifugation. Centrifugation was done in an MSE high-speed 18 centrifuge. Small volumes (under 10 ml) were centrifuged with Teflon adaptors in an 8×50 m head (type 69181).

Measurement of radioactivity. Samples (0.3 ml) were counted in low-background glass vials in 8 ml of scintillator [toluene–2-methoxyethanol (3:2, v/v) containing 80 g of naphthalene and 4 g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen/litre] in a Nuclear–Chicago mark 1 liquid-scintillation spectrometer. An external standard was used to correct for quenching. Simultaneous measurement of ^{3}H and ^{14}C radioactivity was achieved by suitable discrimination in the above machine.

Isolation and incubation in vitro of bovine cornea. Whole cattle eyes were obtained from the slaughter house. They were dissected from the head immediately after the death of the animal and placed in a moist Dewar flask at 37°C. On arrival in the laboratory the corneas were dissected out and stored in the first container of the incubation apparatus described by Handley & Phelps (1972) containing 10 ml of KEI salt–glucose culture medium (Wachtl & Kinsey, 1958) and aerated with O_2+CO_2 (95:5). When about 10 corneas had been dissected out they were transferred to the second container and incubated with the same volume of KEI medium containing [U-^{14}C]glucose (3.2×10^6 c.p.m.; specific radioactivity 196 mCi/mmol). After incubation with the labelled sugar for a fixed period of time the corneas were removed, washed well with 0.9% (w/v) NaCl and blotted dry with filter paper. The epithelium and endothelium were removed by scraping with a stainless-steel scraper. The denuded stroma was then frozen in liquid N_2 and stored at −26°C.

Tissue-space experiments. Corneas (approx. 10 g wet wt.) were incubated with KEI salt–glucose

medium containing 1×10^6c.p.m. of ^3H_2O and 1×10^5c.p.m. of [U-^{14}C]sucrose in the apparatus described (Handley & Phelps, 1972). At various times, corneas (approx. 0.5g wet wt.) were removed from the incubation medium; they were washed in 0.9% (w/v) NaCl and dried between filter paper. The epithelium and endothelium were scraped from the corneas. The denuded stroma was then homogenized with 0.75M-$HClO_4$ (1:3, w/v) and left at 4°C for 12h. The homogenate was neutralized with KOH, centrifuged at 5000*g* for 10min and the supernatants were counted for radioactivity.

Extraction and fractionation of the acid-soluble intermediates of corneal stroma. Whole corneas (approx. 15g), which had been incubated with [U-^{14}C]glucose for a fixed time, were denuded of the epithelium and endothelium before being powdered in portions of 1g in a stainless-steel percussion mortar cooled to the temperature of liquid N_2. The powdered tissue was extracted with $HClO_4$, neutralized and separated on a column (20cm×1.5cm) of Dowex 1 (formate form: 200–400 mesh) as described by Handley & Phelps (1972).

The nucleotides and intermediates of glycosaminoglycan biosynthesis separated on the Dowex 1 (formate form) column were isolated from the fractions on the basis of their position on the elution profile and their spectral ratios (Hurlbert *et al.*, 1954). The fractions corresponding to the intermediates were pooled, deionized and further separated as described by Hardingham & Phelps (1968). The purity of these fractions was confirmed by t.l.c. on polyethyleneimine–cellulose (Randerath & Randerath, 1965). These purified intermediates were analysed and counted for radioactivity as described by Handley & Phelps (1972).

Extraction and fractionation of glycosaminoglycans of corneal stroma. The glycosaminoglycans of corneal stroma were extracted as described by Handley & Phelps (1972) from the residue remaining after treatment with $HClO_4$ and were fractionated on cetylpyridinium chloride–cellulose columns as described by Antonopoulos *et al.* (1961). Dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate were separated from one another by the method of Antonopoulos & Gardell (1963). The purified glycosaminoglycans obtained by chromatography were analysed for uronic acid and hexosamine. The specific radioactivities of their hexosamine and uronic acid residues were determined as described by Handley & Phelps (1972).

Other methods. Electrophoresis and hyaluronidase digestion of the glycosaminoglycan fractions were carried out as described by Handley & Phelps (1972). Samples of the denuded stroma were subjected to histological examination. The tissue was fixed in formaldehyde solution, dehydrated and embedded in paraffin wax. Thin sections were cut, stained with

Table 1. *Cetylpyridinium chloride fractionation of acid glycosaminoglycans of 13g of bovine corneal stroma*

For experimental details see the text. Values given are means±S.D. of four to six experiments. Hexuronic acid was assayed by the carbazole method (°) of Bitter & Muir (1962) and by the orcinol method (°) of Brown (1946) as modified by Khym & Doherty (1952).

Fraction	Hexosamine content (% wet wt.)	Molar ratios: Hexosamine/uronic acid°	Molar ratios: Uronic acid°/uronic acid°	Molar ratios: Sulphate/hexosamine	Identification of hexosamine	Hyaluronidase digestion (%)	Glycosaminoglycan present
1% Cetylpyridinium chloride	71.3±6.5	—	—	0.98	Glucosamine	0	Keratan sulphate
0.3M-NaCl	6.1±2.4	0.93	1.17	0.33		93	
0.25M-$MgCl_2$	7.6±2.0	1.07	0.94	0.56	Galactosamine	100	Chondroitin sulphate
0.5M-$MgCl_2$	12.0±3.1	0.88	0.92	0.66		100	
0.75M-$MgCl_2$	1.7±0.7	0.88	1.14	—		100	
1.25M-$MgCl_2$	0	—	—	—	—	—	—
6M-HCl	0	—	—	—	—	—	—

Haematoxylin and Eosin and examined under the microscope.

Results

Glycosaminoglycans in bovine cornea

Glycosaminoglycans were released from the stroma of bovine cornea by digestion with papain (EC 3.4.4.10) as described by Handley & Phelps (1972). The extraction of bovine corneal stroma released 17.6μmol of hexosamine/g wet wt. of tissue in the form of glycosaminoglycans. This was similar to the value obtained by Anseth (1961) (approx. 15.7μmol/g wet wt.) for bovine cornea. Electrophoresis of the whole glycosaminoglycan extract in 0.2M-zinc sulphate showed two components, the major one corresponding to keratan sulphate and the minor one to chondroitin sulphate. Fractionation on cetylpyridinium chloride–cellulose columns (Antonopoulos *et al.*, 1961) enabled further separation of the glycosaminoglycan extract (Table 1). Electrophoresis of the 1% cetylpyridinium chloride fraction showed it to be made up of one component with the mobility of standard keratan sulphate. After methanolysis of this component with methanolic hydrochloric acid, g.l.c. of the trimethysilyl derivatives revealed the presence of *N*-acetylglucosamine, galactose, fucose and mannose in the molar proportions 5.5:6.0:0.3:0.8. As the polysaccharide was not digested by hyaluronidase, the major glycosaminoglycan of bovine cornea eluted in this fraction was identified as keratan sulphate.

The polysaccharides present in the fractions eluted between 0.3M-NaCl and 0.75M-$MgCl_2$ were digested by hyaluronidase. G.l.c. of the trimethysilyl derivatives showed that the hexosamine present was *N*-acetylgalactosamine. These fractions had different electrophoretic mobilities ranging between those of standard keratan sulphate and chondroitin sulphate. The degree of sulphation (Table 1) varied from a

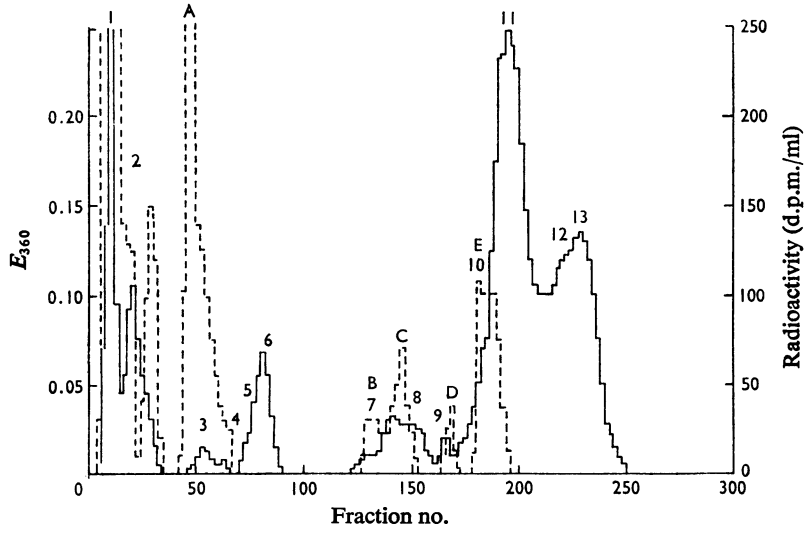


Fig. 1. *Incorporation of radioactive glucose into nucleotides and intermediates of glycosaminoglycan biosynthesis in bovine corneal stroma*

Nucleotide E_{260} (——) and radioactivity (– – – –) profiles of acid-soluble extract of 16g (wet wt.) of bovine corneal stroma that had been incubated for 1h with [U-^{14}C]glucose. The extract was applied to a Dowex 1 (formate form; X8; 200–400 mesh) column (20cm×1.5cm) and eluted with an extended gradient formed with a 200ml sealed mixing vessel and a reservoir containing various concentrations of formic acid and ammonium formate as follows: fractions 1–30, 1M-formic acid; 31–180, 4M-formic acid–0.25M-ammonium formate; 181–210, 4M-formic acid–0.4M-ammonium formate; 211–end, 4M-formic acid–0.8M-ammonium formate. Fractions of volume 5ml were collected. Nucleotide peaks: 1, NAD; 2, AMP; 3, NADP; 4, GMP; 5, UMP; 6, ADP; 7, UDP-*N*-acetylhexosamine; 8, UDP-hexose; 9, GDP; 10, UDP-glucuronic acid; 11, ATP; 12, GTP; 13, UTP. Radioactivity peaks: A, hexose monophosphate and *N*-acetylglucosamine 6-phosphate; B, UDP-*N*-acetylhexosamine; C, UDP-hexose; D, fructose 1,6-diphosphate; E, UDP-glucuronic acid. The components of the fractions were identified as described in the text. No detectable nucleotide material was eluted after fraction no. 250.

sulphate/hexosamine ratio of 0.33 in the 0.3M-NaCl fraction to 0.66 in the 0.5M-$MgCl_2$ fraction. These fractions were ascribed to chondroitin sulphates of different degrees of sulphation. The subfractionation by the method of Antonopoulos & Gardell (1963) of the polysaccharides present in the fractions eluted between 0.25M-$MgCl_2$ and 0.75M-$MgCl_2$ indicated that 80% was present as chondroitin 4-sulphate and 20% as chondroitin 6-sulphate.

Tissue-space experiments

An estimate of the intracellular water volume was obtained from the difference in the total volumes of a standard weight of corneal stroma penetrable by ^3H_2O and [U-^{14}C]sucrose respectively. The space penetrable by sucrose was 72.0±1.0% of the wet weight of the tissue. The space penetrable by ^3H_2O was found to be equivalent to 77.1±0.5% of the wet weight of the tissue. Hence, by difference, the cell water was 5.1±0.75% of the total wet weight of corneal stroma. The values obtained for the intracellular water volume were consistent over a period of time. The histological examination of this tissue confirmed that all the epithelium and endothelium had been removed. These values were used in the calculation of the cellular concentrations of the metabolites and intermediates of glycosaminoglycan biosynthesis in corneal stroma.

Concentrations of the intermediate metabolites in corneal stroma

The elution profile of the neutralized perchloric acid extract of 16g of corneal stroma separated on a column (20cm×1.5cm) of Dowex 1 (formate form) and the corresponding radioactivity profile after incubation for 1h with [U-^{14}C]glucose are shown in Fig. 1. Samples corresponding to individual metabolites were pooled, deionized and freeze-dried as described by Hardingham & Phelps (1968) and Table 2 reports the concentrations of intermediates of glycosaminoglycan observed in bovine corneal stroma.

Time-course of incorporation of [U-^{14}C]glucose into the intermediates of glycosaminoglycan biosynthesis in corneal stroma

The specific radioactivity of each of the precursors on the pathways of glycosaminoglycan biosynthesis was studied as a function of time of incubation with radioactive glucose. The resulting specific radioactivity was plotted against time for the following intermediates: hexose monophosphate, fructose 1,6-diphosphate, *N*-acetylglucosamine 6-phosphate, UDP-hexose, UDP-*N*-acetylhexosamine and UDP-glucuronic acid (Fig. 2). These intermediates were separated on a column of Dowex 1 (formate form)

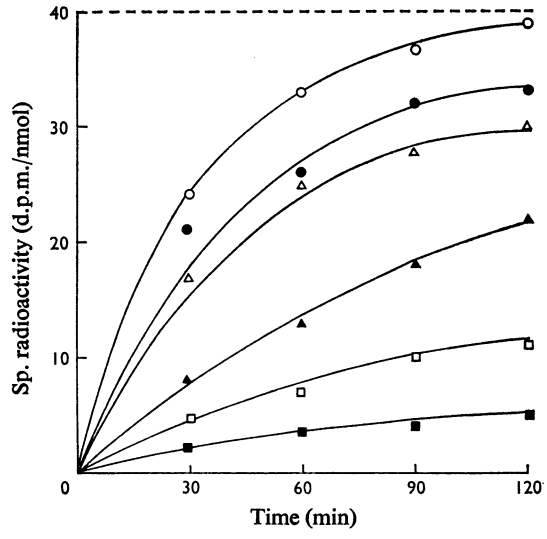


Fig. 2. *Specific radioactivity of intermediates of glycosaminoglycan biosynthesis in bovine corneal stroma*

The results are plotted against time of incubation with [U-^{14}C]glucose. ○, Hexose monophosphate; ●, fructose 1,6-diphosphate; △, *N*-acetylglucosamine 6-phosphate; ▲, UDP-*N*-acetylhexosamine; □, UDP-hexose; ■, UDP-glucuronic acid; ----, glucose (the specific radioactivity remained constant because of the large pool present in the incubation media).

and were further purified by chromatography as described by Hardingham & Phelps (1968) before being assayed and counted for radioactivity. From these results the turnover times of these intermediates were calculated as described by Zilversmit *et al.* (1942) and the rates of synthesis of the intermediates were calculated from their tissue contents (Table 2). The assumed product–precursor relationships were the same as described by Handley & Phelps (1972).

Incorporation of [U-^{14}C]glucose into acid glycosaminoglycans of corneal stroma

The percentage and specific radioactivities of the hexosamine and uronic components of the fractionated glycosaminoglycans after incubation for 1h with [U-^{14}C]glucose were measured (Table 3). From the percentage radioactivity of the hexosamine components the proportional rates of synthesis of the various fractions were calculated.

The ratios of the specific radioactivities of the hexosamine and uronic acid moieties of the fractionated glycosaminoglycans and the ratios of the specific radioactivities of the two immediate precursors of polymer synthesis, UDP-*N*-acetylhexosamine and UDP-glucuronic acid, are similar.

Table 2. *Cellular concentrations and rates of synthesis of intermediates in the biosynthesis of glycosaminoglycans in bovine corneal stroma*

For experimental details see the text. Values given are means±S.D. of four to six experiments. The ratio of the specific radioactivities of UDP-glucose to UDP-galactose was 1:1.1 and the ratio of the specific radioactivities of UDP-*N*-acetylglucosamine to UDP-*N*-acetylgalactosamine was 1:1.1.

Intermediate	Tissue content (nmol/g wet wt.)	Cellular concn. (mM)	Turnover time (min)	Rates of synthesis (nmol/min per g wet wt.)
Hexose monophosphate	30.0±1.5	0.600±0.030	3.65	8.2±0.4
N-Acetylglucosamine 6-phosphate	8.0±1.1	0.160±0.022	3.03	2.6±0.5
UDP-*N*-acetylglucosamine	10.5±1.3	0.211±0.022	6.71	2.3±0.3
UDP-*N*-acetylgalactosamine	4.8±0.9	0.095±0.018		
UDP-glucose	18.2±1.1	0.364±0.022	18.43	1.2±0.1
UDP-galactose	4.3±0.1	0.085±0.002		
UDP-xylose	3.6±0.1	0.072±0.002	—	—
UDP-glucuronic acid	3.0±0.2	0.060±0.004	6.50	0.4±0.1
Fructose 1,6-diphosphate	10.5±1.1	0.210±0.022	2.24	4.7±0.5

Table 3. *Percentage and specific radioactivities of monosaccharides isolated from the glycosaminoglycans of bovine cornea*

For experimental details see the text. Values given are means±S.D. of four to six experiments. The proportional rate of synthesis was calculated from the percentage radioactivity in hexosamine.

Fraction	Percentage radioactivity		Proportional rate of synthesis	Sp. radioactivity (d.p.m./μmol)	
	Hexosamine	Uronic acid		Hexosamine	Uronic acid
1% Cetylpyridinium chloride	39.7±1.5	—	2.9	59.8	—
0.3M-NaCl	23.4±0.9	39.7±0.5	1.7	314.3	123.0
0.25M-$MgCl_2$	13.7±0.8	23.2±0.5	1.0	180.0	56.2
0.5M-$MgCl_2$	19.2±0.8	32.5±0.5	1.4	140.0	52.6
0.75M-$MgCl_2$	2.7±0.3	4.6±0.2	0.2	133.3	57.6

Calculation of the turnover rates of the acid glycosaminoglycans in corneal stroma from ^{14}C labelling

It is assumed in this work that all the UDP-glucuronic acid present in this tissue is incorporated into chondroitin sulphate at the same rate as it is synthesized. Thus the rate of synthesis of the chondroitin sulphate in this tissue will be equal to the rate of synthesis of UDP-glucuronic acid. However, the analysis of glycosaminoglycans present in the stroma showed that there were four chondroitin sulphate fractions present. The rates of synthesis of these chondroitin sulphate fractions were estimated by dividing the rate of synthesis of the UDP-glucuronic acid between these fractions according to the percentage radioactivity found in these fractions. By knowing the tissue content of each of these fractions, the turnover times of the uronic acid-containing fractions were found and, as shown in Table 4, it appears that these chondroitin sulphate fractions are turning over at different rates.

The major glycosaminoglycan of corneal stroma was shown to be keratan sulphate, which does not contain uronic acid. The rate of synthesis of this glycosaminoglycan cannot be determined directly as the rates of incorporation of UDP-*N*-acetylglucosamine or UDP-galactose into keratan sulphate are not known. Therefore the rate of synthesis of keratan sulphate has to be estimated from the proportional rate of synthesis of this fraction. This value was derived from the ratio of the amount of radioactivity observed in the hexosamine components of the fractionated glycosaminoglycans of corneal stroma. As the tissue content of this fraction is known, the turnover rate can be calculated (Table 4). This fraction is turning over more slowly than the uronic acid-containing polymers.

Table 4. *Rates of synthesis and turnover times of glycosaminoglycans in bovine cornea*

For experimental details see the text. Values given are means±S.D. of four to six experiments. The proportional rate of synthesis was calculated from the percentage radioactivity in hexosamine.

Fraction	Tissue content of hexosamine (nmol/g wet wt.)	Proportional rate of synthesis	Rate of synthesis (nmol/h per g wet wt.)	Turnover time (h)
1% Cetylpyridinium chloride	12540±1140	2.9	17.4±0.2	723.2±65.9
0.3M-NaCl	1070± 470	1.7	10.2±0.1	104.8±46.4
0.25M-$MgCl_2$	1330± 350	1.0	6.0±0.1	223.3±58.7
0.5M-$MgCl_2$	2110± 520	1.4	8.4±0.1	251.7±62.9
0.75M-$MgCl_2$	290± 60	0.2	1.2±0.1	251.4±51.7

Discussion

The system described in the present paper and by Handley & Phelps (1972) maintains excised corneas in a physiological state *in vitro*. Mishima & Kudo (1967) showed that the KEI salt–glucose media maintained isolated corneas at their normal thickness for up to 5h. It was important in this work that the intracellular and extracellular spaces were preserved since they were used to calculate the cellular concentrations of the intermediates of glycosaminoglycan biosynthesis. The histological examination of the tissue revealed that there was no contamination of the stroma by epithelium and endothelium cells. The rate of uptake of glucose, calculated from the synthesis of hexose monophosphate from glucose, was found to be 8.2nmol/min per g wet wt. of tissue. The rate of glucose utilization in bovine cornea was reported by Herrmann & Hickman (1948) to be approx. 10.9nmol/min per g wet wt. of tissue. The high rate of synthesis of fructose 1,6-diphosphate found in the present work probably reflects the high rate of glycolysis in this tissue.

Before considering the biosynthesis of glycosaminoglycans in bovine corneal stroma it is necessary to know the types and amounts of these materials present. Keratan sulphate makes up 71%, chondroitin sulphate 17% and chondroitin 6-sulphate 4% of the glycosaminoglycans. We could not determine which isomer of chondroitin sulphate was present in the 0.3M-NaCl fraction, because this fraction appears to be under-sulphated (it was found to have a sulphate/hexosamine ratio of 0.33). The finding of Meyer *et al.* (1953) indicated that keratan sulphate occurred together with chondroitin sulphate and chondroitin in bovine cornea, but other authors have favoured a polydispersed chondroitin sulphate fraction of various degrees of sulphation (Anseth & Laurent, 1961; Berman & Saliternik-Givant, 1966; Fransson & Anseth, 1967) and there has been no conclusive report of the isolation of chondroitin from this tissue.

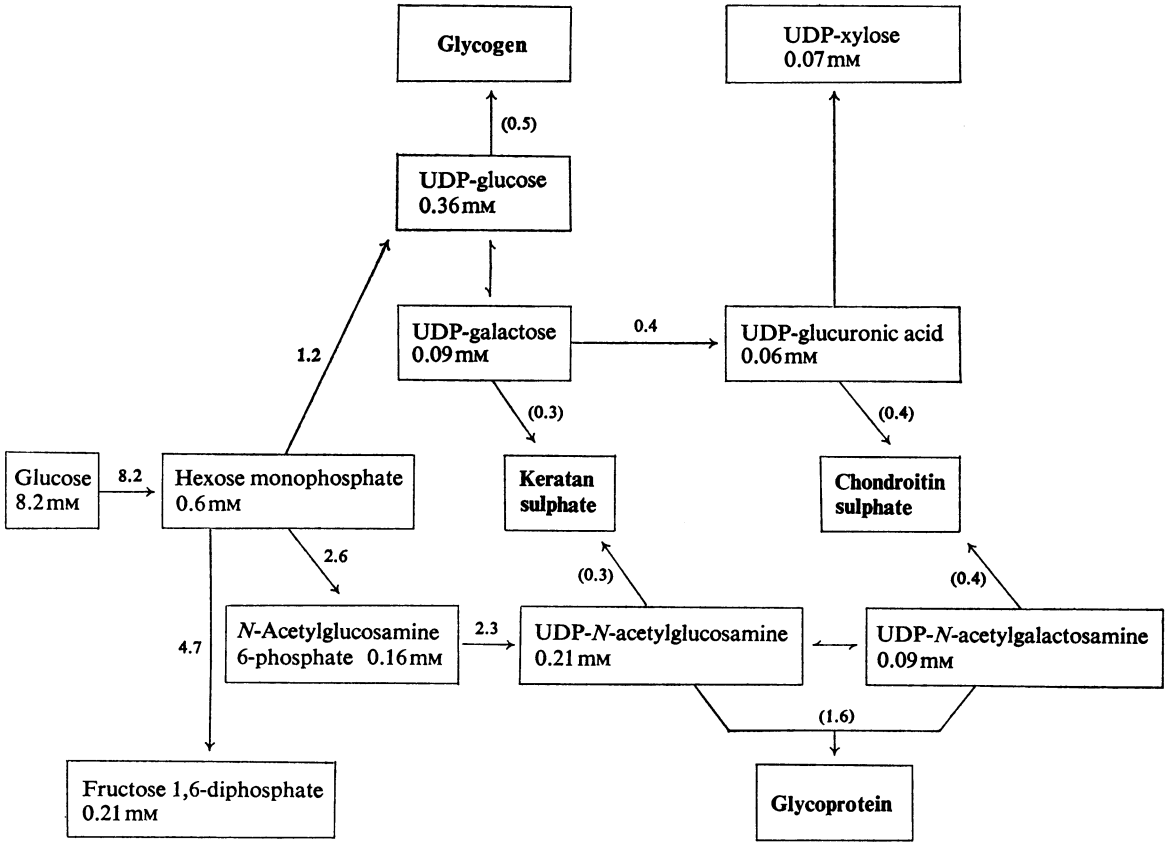
To determine the cellular concentration of intermediates and metabolites, chromatographic separation of the neutralized perchloric acid extracts on Dowex 1 (formate form) was used. The elution profile of the acid-soluble extract of corneal stroma showed a similar distribution to that for neonatal rat epiphysial cartilage (Handley & Phelps, 1972) and neonatal rat skin (Hardingham & Phelps, 1968). The high concentrations of ATP and UTP confirm that the incubation system sustains this tissue *in vitro*.

From the variation of specific radioactivity of these intermediates with time, the flux of radioactive label from glucose through each of the intermediates of glycosaminoglycan biosynthesis can be studied. The cellular concentrations of the intermediates and the fluxes between the metabolite pools are summarized in Scheme 1. All the fluxes are assumed to be unidirectional. It is assumed in this work that all corneal stroma cells are synthesizing glycosaminoglycans and that there are common pools of precursors within these cells. The rates of synthesis of hexose monophosphate, fructose 1,6-diphosphate, *N*-acetylglucosamine 6-phosphate, UDP-hexose and UDP-*N*-acetylhexosamine and UDP-glucuronic acid were all calculated from the specific radioactivity–time curves of the purified components (Zilversmit *et al.*, 1942). Glucuronic acid and *N*-acetylgalactosamine are incorporated in equimolar amounts into chondroitin sulphate. Therefore the rates of incorporation of UDP-*N*-acetylgalactosamine and UDP-glucuronic acid into chondroitin sulphate will be the same. Similarly, because UDP-*N*-acetylglucosamine and UDP-galactose are incorporated in equimolar amounts into keratan sulphate, the rates of incorporation were assumed to be the same and were calculated from the relative rates of synthesis of the polymers. The UDP-xylose concentration in this tissue was considerably greater than those observed in other tissues studied by this method, and has the effect of introducing an uncertainty into the rate of incorporation of UDP-glucuronic acid into chondroitin sulphate since a significant portion of this nucleotide

sugar is diverted into the production of UDP-xylose. Because the rate of synthesis of UDP-xylose from UDP-glucuronic acid was not determined the rates of synthesis of keratan sulphate and chondroitin sulphate as shown in Scheme 1 are all maximum rates and the rates of synthesis of glycogen and glycoproteins will be minimum rates. A high rate of glycolysis in this tissue is suggested by the high rate of fructose 1,6-diphosphate synthesis. From the difference between the rate of synthesis of UDP-hexose from hexose monophosphate and the rate of utilization of UDP-hexose to UDP-glucuronic acid and keratan sulphate, the rate of incorporation of UDP-hexose into glycogen can be calculated. It appears that the rate of synthesis of glycogen in this tissue is comparatively low. But the rate of incorporation of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine into glycoprotein suggests that corneal stroma is synthesizing large amounts of these materials, as UDP-*N*-acetylglucosamine is additionally a precursor of *N*-acetylneuraminic acid, a major sugar component of glycoproteins.

It is possible to draw some conclusions in respect of the control of glycosaminoglycan biosynthesis from the specific activities and pool sizes of the various precursors.

(1) The ratio of the epimers of UDP-hexose and UDP-*N*-acetylhexosamine in bovine corneal stroma are very similar to the free equilibrium of the two epimerases, UDP-glucose 4-epimerase (EC 5.1.3.2) and UDP-*N*-acetylglucosamine 4-epimerase (EC 5.1.3.7) (Leloir, 1951; Glaser, 1959). These ratios are



Scheme 1. *Diagrammatic summary of the net rates of synthesis and pool size of the intermediates of glycosaminoglycan biosynthesis in bovine corneal stroma*

The values refer to the net rates of synthesis observed and are expressed as nmol/min per g wet wt. of tissue (values in parentheses are calculated rates of synthesis). The concentrations of the intermediates are expressed in terms of cell water.

close to those reported for neonatal rat skin (Hardingham & Phelps, 1968) and for neonatal rat epiphysial cartilage (Handley & Phelps, 1972). It appears that these two epimerases in these different tissues are holding their equilibrium ratios under different conditions of nucleotide sugar demand, since neonatal rat skin is biosynthesizing a glucosaminoglucuronan (hyaluronic acid), epiphysial cartilage a galactosaminoglucuronan (chondroitin sulphate) and bovine corneal stroma a non-glucuronic acid-containing polymer (keratan sulphate). The value of the ratios of the specific radioactivities of the epimers in these tissues suggest that the epimerization steps are rapid and therefore unlikely to be rate-limiting.

(2) The enzyme UDP-glucose dehydrogenase (EC 1.1.1.22), converting UDP-glucose into UDP-glucuronic acid, was demonstrated by Neufeld & Hall (1965) to be inhibited by UDP-xylose (Gainey & Phelps, 1972). The latter reported that the concentration of UDP-xylose for 50% inhibition of the enzyme in bovine cornea was approx. 11–12 μM for the physiological UDP-glucose concentration of 0.380mM. In stroma where the values of UDP-xylose are 0.072mM, the activity of this enzyme in this tissue would be more than 90% inhibited. This is significant in view of low concentration of uronic acid-containing polymer in corneal tissue.

(3) The enzyme L-glutamine D-fructose 6-phosphate aminotransferase (EC 2.6.1.16) has been shown by Kornfeld *et al.* (1964) to be subjected to feedback inhibition by UDP-*N*-acetylglucosamine. If the enzyme in corneal stroma resembles that in rat liver, the work of Winterburn & Phelps (1971) indicates it is about 75% inhibited.

From the time-dependence of the radioactivity emerging in the complete polymers, the turnover rates of the glycosaminoglycans of corneal stroma can be calculated. Chondroitin sulphate turns over in about 250h and keratan sulphate in about 720h. These values compare well with those reported by Schiller *et al.* (1956) for chondroitin 4-sulphate in adult rabbit skin and by Davidson & Small (1963) for keratan sulphate and chondroitin 6-sulphate in rabbit nucleus pulposus. These results indicate that the glycosaminoglycans of adult corneal stroma are turning over more slowly than those in neonatal tissue (Hardingham & Phelps, 1970*a*; Handley & Phelps, 1972).

When the cellular concentrations and rates of synthesis of the intermediates of glycosaminoglycan biosynthesis in corneal stroma are compared with those reported in neonatal rat skin (Hardingham & Phelps, 1970*a*) and in neonatal rat epiphysial cartilage (Handley & Phelps, 1972), it is seen that the rates of synthesis of intermediates are similar, although these tissues are of different species and differ in ages and in the spectrum of glycosaminoglycans produced. It would also appear that the pathway of glycosaminoglycan biosynthesis is controlled by demand, that is, the lowering of concentration of an immediate donor of polymer synthesis on incorporation into a glycosaminoglycan lifts the inhibition on the controlling steps of the pathway of synthesis of these donors, causing more to become available for polymer synthesis. It is apparent, from the detailed analysis of the flow of radioisotopic label through the intermediates in these three tissues, that the control exerted on the nucleotide sugar pathway is explainable in the forms of known feedback inhibition by end-products on the initial enzyme reactions (Kornfeld *et al.*, 1964).

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