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Synthesis of hyaluronate in differentiated teratocarcinoma cells

Mechanism of chain growth

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Hyaluronate could be labelled *in vivo* with [^{32}P]phosphate. [^{32}P]UDP in an α-glycosidic linkage constituted the reducing end of membrane-bound hyaluronate. The UDP is liberated during further chain elongation, indicating that chain growth occurs at the reducing end. [^{3}H]Uridine could be incorporated into hyaluronate during synthesis on the isolated membraneous fraction from [^{3}H]UDP-GlcNAc and [^{3}H]UDP-GlcA, confirming the identification of UDP as a constituent of membrane-bound hyaluronate. These results led to a model of hyaluronate chain elongation at the reducing end by alternate addition of the chains to the substrates. Membrane-bound pyrophosphatases or 5′-nucleotidase are suggested as modulators of hyaluronate synthesis.

The mechanism of hyaluronate synthesis does not follow the same laws as that of other known glycosaminoglycans (Rodén & Horowitz, 1978; Kleine, 1981). Attempts to influence hyaluronate synthesis by chemicals which alter the synthesis of other glycosaminoglycans failed. A search for lipid intermediates was unsuccessful (Ishimoto & Strominger, 1967). Tunicamycin, which inhibits the synthesis of dolichol phosphate precursors for glycopeptides, had no influence on hyaluronate synthesis (Hart & Lennarz, 1978). Xylosides, which interfere with the synthesis of proteoglycans, did not affect hyaluronate synthesis (Hopwood & Dorfman, 1977). Although peptides or proteins have been claimed to be covalently bound to purified hyaluronate (Tagakaki & Toole, 1981), their role in its synthesis remained doubtful, since hyaluronate can be synthesized *in vivo* in the absence of protein synthesis (Mapleson & Buchwald, 1981) and no peptide could be found on hyaluronate synthesized *in vitro* (Sugahara *et al.*, 1979). Inhibition studies with drugs (Kleine, 1978) and incorporation experiments with radioactive precursors (von Figura *et al.*, 1973) indicated that proteoglycans and hyaluronate have different mechanisms of biosynthesis and use different precursor pools of UDP-GlcNAc and UDP-GlcA.

As described in the preceding paper (Prehm, 1983), kinetic studies on hyaluronate synthesis and its stimulation by phosphate esters indicated that hyaluronate is anchored by a phosphate ester bridge to membranes during synthesis. The chains of hyaluronate probably grow at the reducing end by a mechanism which is independent of added primers. The chemical nature of the reducing end of hyaluronate, which holds it to the membrane, is still a matter of controversy. Claims of arabinose as a constituent and possible termination of the chains (Stary *et al.*, 1965; Varma *et al.*, 1977) could not be verified (Katzman, 1974) and it is still controversial whether protein is bound covalently to hyaluronate (Swann, 1968; Varma *et al.*, 1974; Tagakaki & Toole, 1981).

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Materials

[5-^{3}H]Uridine 5′-triphosphate (15.1 Ci/mmol) and [^{32}P]phosphate (carrier free) were from Amersham International; ^{125}I and uridinediphospho-*N*-acetyl-[^{3}H]glucosamine (66 Ci/mmol) were from New England Nuclear; inorganic pyrophosphatase (yeast; 500 units/mg), uridinediphosphoglucose dehydrogenase (*Escherichia coli*; 0.07 units/mg) and yeast enzyme concentrate type II were from Sigma. Other materials were as described in Prehm (1983).

Methods

Polyacrylamide-gel electrophoresis and cellulose acetate electrophoresis were performed as described [Laemmli (1970) and Fransson & Rodén (1967) respectively]. Iodination was by the chloramine-T method as described by Greenwood *et al.* (1963) and by the Bolton & Hunter procedure (1973). Ion-exchange chromatography was conducted on columns (7 cm × 0.8 cm) of DEAE-Sephacel in

10mM-Tris/HCl buffer, pH8.4, containing 0.1% Emulphogen BC-710 and a linear gradient of 0–0.6M-NaCl (Winterbourne & Mora, 1978). Hydrophobic chromatography on octyl-Sepharose was performed as described by Kjellen *et al.* (1981).

Isolation of [^{32}P]phosphate-labelled hyaluronate

For isolation of ^{32}P-labelled hyaluronate, 2×10^7 cells of the strain 756 (a mesenchymal-like derivative of teratocarcinoma cells kindly provided by Dr. R. Kemmler) were seeded on 5.8g of microcarrier beads (Biosilon, NUNC, Castrup, Denmark) with 1500cm^2 surface area and grown in 150ml suspension culture for 5 days with changes of medium every 24h. The normal Dulbecco's medium with 10% (v/v) foetal calf serum was then substituted by medium in which the phosphate buffer was omitted and serum to which 15mCi of [^{32}P]phosphate had been added. After 4h, 30ml of serum was added and incubation was continued for 5h. The medium was decanted, the cells were washed twice with 100ml of phosphate-buffered saline and suspended in 100ml of the same buffer. The cells were disrupted by freezing and thawing of the suspension and the beads were sedimented for 5min at 800*g*. From the supernatant the membranes were isolated by the method of Brunette & Till (1971). The membrane preparation was solubilized at 100°C for 3min in 300μl of phosphate-buffered saline containing 4% (w/v) SDS. Undissolved material was sedimented for 3min at 10000*g* and the supernatant was applied to a Sepharose CL2B column (50cm × 0.8cm) and eluted with phosphate-buffered saline containing 0.1% SDS. The excluded high-molecular-weight fraction was applied to DEAE-Sephacel and eluted as described above.

Identification of ^{32}P-labelled compounds

[^{32}P]Phosphate-labelled compounds liberated from [^{32}P]hyaluronic acid by acid hydrolysis or incubation with membranes were separated by t.l.c. on cellulose with propan-1-ol/ammonia (sp. gravity 0.9)/water (6:3:1, by vol.) or *n*-propyl acetate/90% formic acid/water (11:5:3, by vol.) or by thin layer electrophoresis on cellulose in 0.1M-pyridine acetate buffer, pH3.5, at 50V/cm for 60min (Bieleski, 1965), or by ion-exchange thin layer electrophoresis on polyethyleneimine cellulose with 1M-LiCl as solvent (Randerath & Randerath, 1964). The radioactive compounds were visualized by autoradiography and the unlabelled nucleotides by their fluorescence under u.v. light (emission peak 265nm).

Synthesis of [5-^{3}H]UDP-GlcA

[5-^{3}H]UTP (330μl; 300μCi) was evaporated to dryness and incubated with 20μl of glucose 1-phosphate (100mM), 10μl of ATP (100mM), 20μl of $MgCl_2$ (50mM), 5μl of inorganic pyrophosphatase (5 units), and 50μl of yeast enzyme concentrate (1mg) in 0.2M-Tris/HCl buffer, pH7.5, for 20min at 37°C. The [5-^{3}H]UDP-Glc was isolated by t.l.c. on cellulose [solvent: ethanol/aqueous 1M-ammonium acetate, pH5.5 (13:7, v/v)], visualized by autoradiography and recovered by scraping off the radioactive area into a centrifuge tube. The cellulose was suspended in water, centrifuged and the supernatant was evaporated to dryness. The recovered [5-^{3}H]UDP-Glc was disssolved in 80μl of water and incubated with 12μl of 1M-Tris/HCl buffer, pH8.7, 12μl of NAD^+ (20mM) and 12μl of uridine-diphosphoglucose dehydrogenase (0.1 unit) for 12h at 37°C. The mixture was separated by t.l.c. The radioactive product was visualized by autoradiography and eluted with water.

Synthesis of [5-^{3}H]UDP-GlcNAc

[5-^{3}H]UTP (200μl; 300μCi) was dried by evaporation and incubated with 20μl of *N*-acetylglucosamine 1-phosphate (100mM), 10μl of ATP (100mM), 20μl of $MgCl_2$ (50mM), 5μl of inorganic pyrophosphatase (5 units) and 50μl of yeast enzyme concentrate (1mg) in 0.2M-Tris/HCl buffer, pH7.5, for 20min at 37°C. The enzyme reaction was stopped by heating for 2min at 100°C and the solution was applied to cellulose t.l.c. in ethanol/1M-ammonium acetate, pH5.5, (13:7, v/v). The radioactive product was visualized by autoradiography and eluted with water. The eluate was concentrated by evaporation.

Incorporation of [^{3}H]uridine into hyaluronate from [^{3}H]UDP-GlcNac

Membranes (20mg) were incubated in 100μl of 40mM-phosphate buffer, pH6.7, with 5μM-UDP-GlcA, 8μM-[^{3}H]UDP-GlcNAc (2×10^7c.p.m.), 5mM-ATP and 10mM-$MgCl_2$ for 6h at 37°C. The reaction was stopped by addition of 5μl of 20% (w/v) SDS and heating for 3min at 100°C. The mixture was applied to DEAE-Sephacel. The column was eluted with a linear gradient of 0–0.5M-NaCl in 10mM-Tris/HCl, pH8.4, containing 0.1% SDS. Fractions (2ml) of the eluate were collected and 0.4ml aliquots were used for determination of the radioactivity.

Isolation of [^{3}H]uridine–hyaluronate from [^{3}H]-UDP-GlcA

Membranes (15mg) were incubated in 50μl of 40mM-phosphate buffer, pH6.7, with 8μM-[^{3}H]-UDP-GlcA, 5μM-UDP-GlcNAc, 5mM-ATP and 10mM-$MgCl_2$ for 2h at 37°C. Labelled hyaluronate was isolated as described above.

Results

Properties of membrane-bound hyaluronate

Membranes were isolated from differentiated

teratocarcinoma cells and incubated with substrate for hyaluronate synthesis in order to charge all possible primers. The membranes were then ^{125}I-iodinated by the chloramine-T method or by the Bolton–Hunter reagent. From this incubation mixture hyaluronate was isolated by gel filtration and ion-exchange chromatography. The isolated hyaluronate did not contain any ^{125}I radioactivity.

The Triton X-114 separation method which separates membrane constituents from soluble proteins (Bordier, 1981), left hyaluronate in the water phase.

Octyl-Sepharose did not adsorb membrane-bound hyaluronate from a 3 M-NaCl solution.

Isolation of ^{32}P-labelled hyaluronate

Membranes were prepared from cells labelled with [^{32}P]phosphate, solubilized by SDS at 100°C for 3 min, applied to a Sepharose CL 2B column and eluted with phosphate-buffered saline containing 0.1% SDS (Fig. 1). The excluded fraction was applied to ion-exchange chromatography after addition of 1 mg of hyaluronate as carrier (Fig. 2). The fractions eluting as hyaluronate at 300 mM-NaCl were dialysed and characterized. They were digested with mammalian hyaluronidase. Fig. 3 shows an autoradiogram of a 10% (w/v) SDS/polyacrylamide gel of the sample before and after hyaluronidase treatment. The degraded ^{32}P-labelled product migrated with the dye front. The ^{32}P radioactivity eluted from the Sepharose CL 2B column could be precipitated with bovine serum albumin (Dorfman, 1955). Treatment with hyaluronidase prevented the precipitation. The ^{32}P-labelled preparation could not be degraded by deoxyribonuclease I, or ribonuclease A or Pronase and was insoluble in butan-1-ol or chloroform, excluding the possibility of contaminating nucleic acids, proteins or phospholipids.

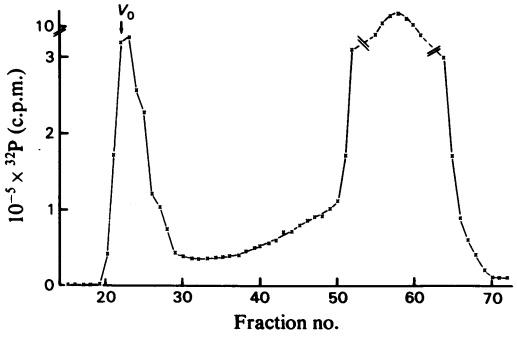

Fig. 1. *Gel chromatography of ^{32}P-labelled hyaluronate on Sepharose CL 2B*
Details were as described in the Materials and methods section.

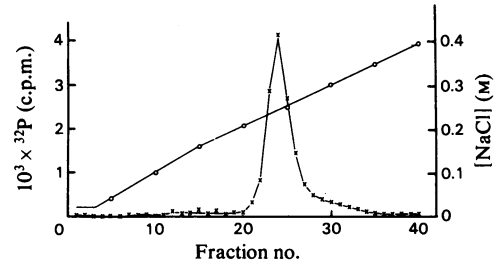

Fig. 2. *DEAE-Sephacel ion-exchange chromatography of [^{32}P]hyaluronate*
Details were as described in the Materials and methods section. ×, ^{32}P radioactivity; O, [NaCl].

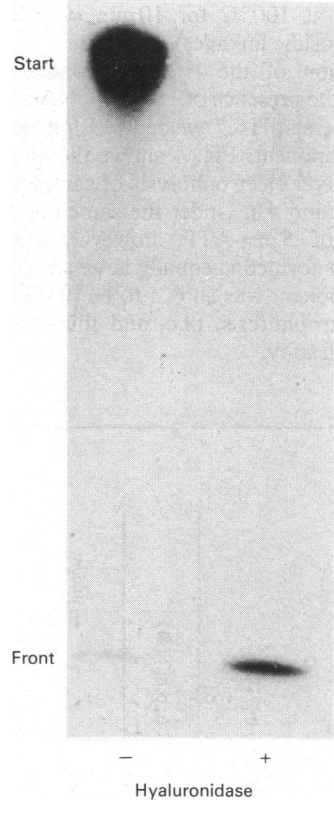

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of [^{32}P]-hyaluronate before and after hyaluronidase digestion*
The radioactive fractions from the DEAE-Sephacel column were collected and dialysed against water. An aliquot was adjusted to 20 mM-sodium acetate, pH 5.3, and incubated with 40 μg of hyaluronidase from bovine testis for 6 h at 37°C. An untreated and a hyaluronidase digested sample was loaded onto a 10% SDS/polyacrylamide gel. After electrophoresis the gel was dried and exposed to an X-ray film.

Identification of the ^{32}P-labelled compound present in membrane-bound hyaluronate

After hyaluronidase treatment the ^{32}P-labelled product migrated in the dye front of a 10% SDS/polyacrylamide gel (Fig. 3). This material was insoluble in water and could not be extracted by butan-1-ol. It was adsorbed completely to activated charcoal, which is characteristic of nucleotides (Crane & Lipmann, 1953).

The [^{32}P]hyaluronate was treated with 0.2M-NaOH at 37°C for 30min, with 0.2M-HCl at 100°C for 10min, and with 1M-HCl at 23°C for 30min. The reaction products were separated by thin layer electrophoresis on cellulose and visualized by autoradiography (Bieleski, 1965). The [^{32}P]hyaluronate was stable towards alkaline degradation and towards 1M-HCl at 23°C [which cleaves β-glycosidic phosphate esters (Leloir & Cardini, 1957; O'Brien, 1964)]. It was, however, partially degraded by 0.2M-HCl at 100°C for 10min (which cleaves α- and β-glycosidic linkages).

Incubation of the [^{32}P]hyaluronate with membranes in the presence of UDP-GlcNAc, UDP-GlcA and $MgCl_2$ at pH6.7 yielded [^{32}P]phosphate as the major component. Fig. 4 shows the autoradiogram of a thin layer electrophoresis of samples withdrawn at 0, 1, 2, and 4h. Under the same condition in the presence of 5mM-ATP, however, another component was formed in equally large amounts (Fig. 4). This component was shown to be [^{32}P]UDP by thin layer electrophoresis, t.l.c. and thin-layer-exchange chromatography.

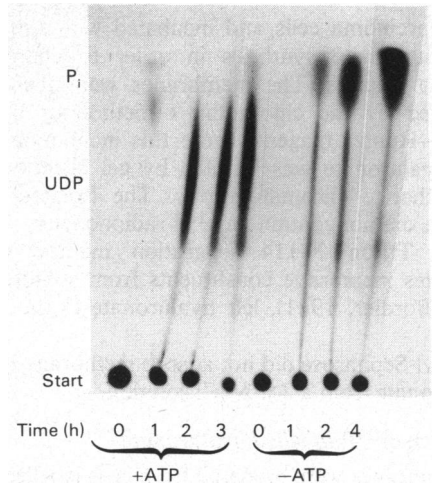

Fig. 4. *Thin layer electropherogram of* [^{32}P]*hyaluronate incubated with membranes, UDP-GlcNAc, UDP-GlcA and* $MgCl_2$ *in the presence and absence of 5mM-ATP*
[^{32}P]Hyaluronate isolated from the DEAE-Sephacel column was dialysed against water, concentrated by evaporation and 10μl aliquots were incubated with 10μl of a membrane preparation (2.5mg/ml) and 50μl of 10mM-$MgCl_2$/4mM-dithiothreitol/8μM-UDP-GlcA/166μM-UDP-GlcNAc/40mM-phosphate buffer, pH6.7, with and without 5mM-ATP. At the times indicated 10μl of the incubation mixture was withdrawn and frozen until they were applied to thin layer electrophoresis, as described in the Materials and methods section.

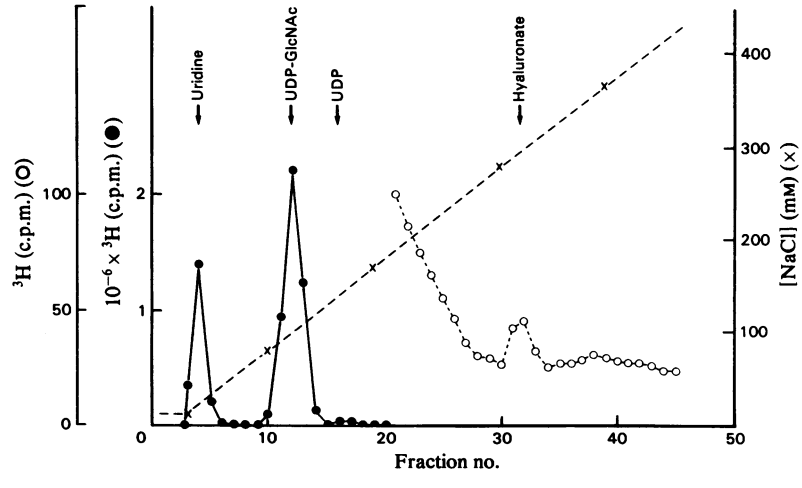

Fig. 5. *DEAE-Sephacel ion-exchange chromatography of solubilized membranes which had been incubated with* [^3H]*UDP-GlcNAc, UDP-GlcA and* $MgCl_2$ *in 40mM-phosphate buffer, pH6.7*
Details were as described in the Materials and methods section. ● and O, ^3H radioactivity; ×, [NaCl].

These experiments indicated that UDP could be a constituent of membrane-bound hyaluronate, located at its reducing end in α-glycosidic linkage. Such α-glycosidic linkages also occur in the substrates UDP-GlcNAc and UDP-GlcA.

Incorporation of uridine into hyaluronate in vitro

For incorporation into hyaluronate the substrates [^{3}H]UDP-GlcNAc and [^{3}H]UDP-GlcA were synthesized from [^{3}H]UTP and GlcNAc-P and Glc-P, respectively, by modified procedures of Rao & Mendicino (1978) and Strominger *et al.* (1957).

A membrane preparation was incubated with [^{3}H]UDP-GlcNAc under conditions of maximal synthesis for 2h. The reaction was stopped with SDS and heating for 3min at 100°C and the mixture was separated by ion-exchange chromatography (Fig. 5). At the elution position of hyaluronate at 300mM-NaCl a small but distinct peak of radioactivity was noted. It was collected, dialysed against water and concentrated by evaporation. The fraction was further characterized by gel filtration on Sepharose CL2B before and after treatment with bacterial hyaluronidase (Fig. 6). The radioactive material was eluted from Sepharose CL2B as a broad peak. Dissociation in 4M-guanidinium chloride and gel filtration in 4M-guanidinium chloride/40mM-phosphate buffer, pH6.4, did not alter the elution profile. However, the radioactive material could be degraded by bacterial hyaluronidase (Fig. 6). The fraction migrated on cellulose acetate electrophoresis together with authentic hyaluronate.

This experiment showed that [^{3}H]uridine can be incorporated into newly synthesized hyaluronate. In the absence of $MgCl_2$ or UDP-GlcA this incorporation was not observed, indicating that the reaction is specifically related to hyaluronate synthesis. Similarly, [^{3}H]uridine could be incorporated into hyaluronate from [^{3}H]UDP-GlcA and UDP-GlcNAc (Fig. 7).

The incorporated radioactivity corresponded to 13×10^{-15}mol from UDP-GlcNAc and 52×10^{-15}mol from UDP-GlcA.

Discussion

The properties of the membrane-bound hyaluronate indicated that it did not contain any protein primer as a membrane-intercalated anchor. Previous experiments suggested that the growing hyaluronate chain could be anchored to the membrane via a phosphate ester bridge (Prehm, 1983). For the identification of its chemical nature a mesenchymal-like derivative of teratocarcinoma cells was grown on microcarrier beads and labelled with [^{32}P]-phosphate. When cells were fed *in vivo* with [^{32}P]phosphate, [^{32}P]hyaluronate could be isolated. The membrane-bound hyaluronate was shown to contain UDP in an α-glycosidic linkage, which also occurs in the substrates UDP-GlcNAc and UDP-

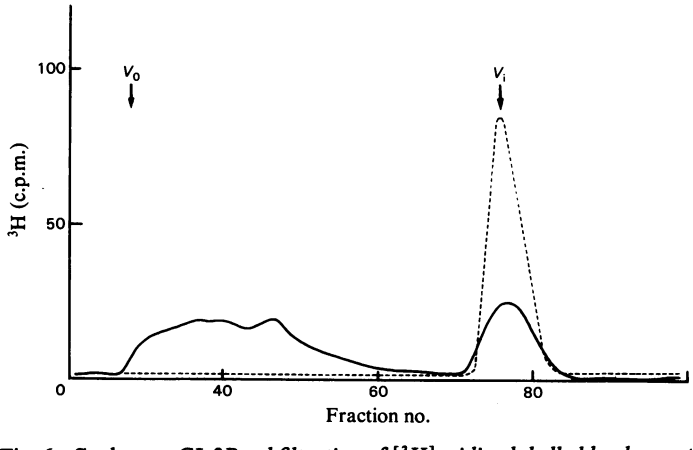

Fig. 6. *Sepharose CL 2B gel filtration of* [^{3}H]*uridine labelled hyaluronate*

To the hyaluronate-containing fractions from the DEAE-Sephacel column was added 50μg of carrier hyaluronate. Fractions were dialysed against water and concentrated by evaporation to 150μl. A 50μl aliquot was directly applied to a Sepharose CL2B column (0.8cm × 50cm) and eluted with phosphate-buffered saline. Fractions (0.3ml) were collected and their radioactivity was determined by liquid-scintillation counting. Another 50μl was adjusted to 20mM-sodium acetate, pH5.5, and incubated with 5 NF units of bacterial hyaluronidase for 1h at 37°C. The incubation mixture was applied to the same Sepharose CL2B column. Elution patterns before (——) and after (– – –) hyaluronidase digestion are shown.

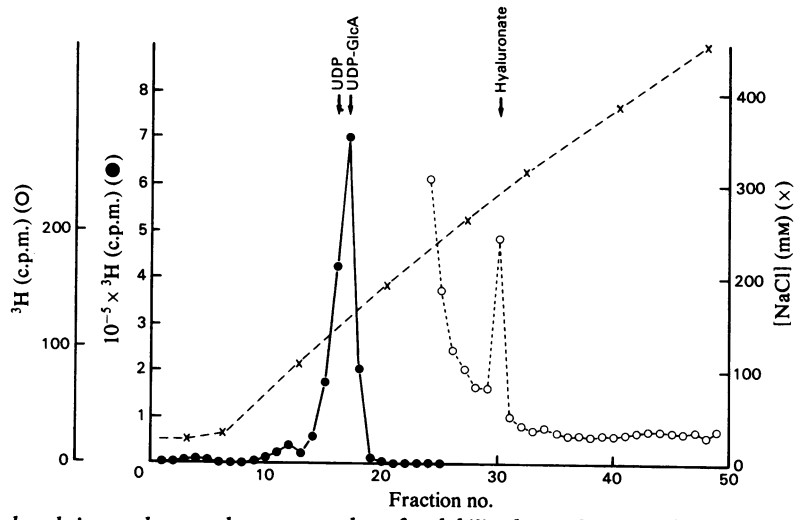

Fig. 7. *DEAE-Sephacel ion-exchange chromatography of solubilized membranes which had been incubated with [^{3}H]UDP-GlcA, UDP-GlcNAc and $MgCl_2$ in 40 mM-phosphate buffer, pH 6.7*
For details see the Materials and methods section. ● and O, ^{3}H radioactivity; ×, [NaCl].

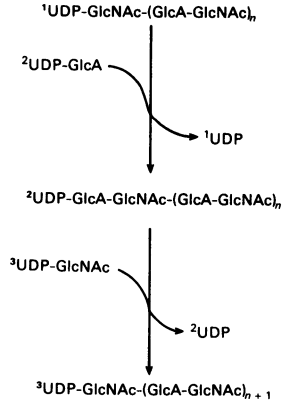

Fig. 8. *Proposed mechanism of hyaluronate synthesis*
Hyaluronate chains are elongated by alternate addition of their reducing end to the substrates UDP-GlcNAc and UDP-GlcA with liberation of UDP. Chain initiation occurs with the substrate itself ($n = 0$).

GlcA. The substrate could therefore constitute the reducing end of the membrane-bound hyaluronate. When the membranes were incubated with [^{32}P]-hyaluronate under the conditions of hyaluronate synthesis, they liberated either [^{32}P]phosphate or [^{32}P]UDP in the absence or presence of ATP, respectively. The liberation of [^{32}P]UDP during chain elongation suggests that the chains grew at the reducing end by alternate addition of the chain UDP-GlcNAc and UDP-GlcA.

Extensive purification procedures could not settle the question of covalently bound protein (Swann, 1968; Varma *et al.*, 1974; Takagaki & Toole, 1981), arabinose (Stary *et al.*, 1965; Katzman, 1974; Varma *et al.*, 1977) or other constituents (Wardi *et al.*, 1969; Varma *et al.*, 1975), since it was practically impossible to purify such a large molecule free of contamination. Therefore, the same reservations also applied for the identification of UDP as a covalent component of hyaluronate. An unequivocal identification was required for the component at the reducing end of membrane-bound hyaluronate. Direct incorporation of [^{3}H]uridine from UDP-GlcNAc and UDP-GlcA into nascent hyaluronate provided this evidence. The finding that [^{3}H]uridine could be incorporated into hyaluronate from either [^{3}H]UDP-GlcNAc or [^{3}H]UDP-GlcA eliminated the possibility that an intermediate disaccharide such as UDP-GlcNAc-GlcA or UDP-GlcA-GlcNAc served as precursor for hyaluronate synthesis.

Together with the results described in the preceding paper (Prehm, 1983), the mechanism of hyaluronate synthesis can be formulated as illustrated in Fig. 8. This mechanism of hyaluronate synthesis does not require a primer molecule other than the substrates themselves. It is also in agreement with the finding that new chains are constantly initiated during active synthesis simultaneously with chain elongation. Hyaluronate synthesis ceases when the substrate is depleted by the synthase or by degradation with pyrophosphatases or 5′-nucleotidase. The unequivocal verification of this scheme

requires the identification of uridine in the developing chain in a 1:1 stoichiometry of uridine:hyaluronate.

A pyrophosphatase or 5'-nucleotidase could also be responsible for hyaluronate shedding from membranes and thus determine its chain length. A pyrophosphatase has already been identified in several cell lines (Bischoff *et al.*, 1975; Touster *et al.*, 1970; Mardh & Vega, 1980; Haugen & Skrede, 1977; Sawicka *et al.*, 1979); however, its physiological role remained unknown (Abney *et al.*, 1976). Synthesis and secretion of hyaluronate vary drastically between cell lines of different origins. Several virus-transformed cell lines have been shown to produce and secrete elevated amounts of hyaluronic acid (Hopwood & Dorfman, 1977; Mikuni-Takagaki & Toole, 1979). Independent of this observation a lack of a pyrophosphatase (Sela *et al.*, 1972) has been detected in these cell lines as a consequence of transformation. A close correlation has also been observed between the regulation of hyaluronic acid synthesis and alkaline phosphatase activity (Koyama & Ono, 1972). Similarly, the precise function of membrane-bound 5'-nucleotidase remained ill-defined (Van den Berghe *et al.*, 1977; Drummond & Masanobu, 1971). Synovial cells from patients with rheumatoid arthritis produce hyaluronate of lower molecular weight (Castor *et al.*, 1971; Mankin, 1979). This may also be related to the disturbance of phosphate metabolism leading eventually to deposition of hydroxyapatite crystals (Howell, 1981).

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