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Synthesis of hyaluronate in differentiated teratocarcinoma cells

Characterization of the synthase

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Differentiation of teratocarcinoma cells led to induction of hyaluronate synthesis. The synthase was recovered in the membrane fraction of cell lysates. Hyaluronate was synthesized at the membranes and was then released as a soluble product. The synthase could be stimulated by a variety of phosphate esters which prevented the degradation of the substrates UDP-GlcNAc and UDP-GlcA and the release of the growing hyaluronic acid chain from the membrane. Hyaluronidases or oligosaccharides derived from hyaluronate did not affect the synthesis. The chains grew at a rate of 60 repeating units/min. Continuous new chain initiation occurred during prolonged synthesis. Digestion of pulse–chase-labelled hyaluronate with β-*N*-acetylglucosaminidase and β-glucuronidase showed that the chains grew at the reducing end.

Hyaluronate is synthesized and secreted by many differentiated cells. Hyaluronate synthesis is modulated during the migration of mesenchymal cells (Toole, 1972), the invasion of carcinoma cells (Toole *et al.*, 1979), differentiation of teratocarcinoma cells (Prehm, 1980), morphogenesis of embryonic tissue (Toole *et al.*, 1977), and virus transformation of fibroblasts (Hopwood & Dorfman, 1977) and chondrocytes (Mikuni-Takagaki & Toole, 1979). The hyaluronate synthase activity is dependent on the cell density (Tomida *et al.*, 1975; Prehm, 1980; Hronowski & Anastassiades, 1980) and the cell cycle (Tomida *et al.*, 1975) in normal fibroblasts. The biosynthesis of hyaluronate has been studied in some detail in *Streptococcus* (Stoolmiller & Dorfman, 1969; Sugahara *et al.*, 1979), but its mechanism has not been clarified (Rodén & Horowitz, 1978; Kleine, 1981).

Materials and methods

Materials

Hyaluronidase (EC 3.2.1.35) chromatographically purified from bovine testis (13000 NF units/mg), ribonuclease A (EC 3.1.4.22) from bovine pancreas (60 Kunitz units/mg), trypsin (EC 3.4.21.4) from pig pancreas (15310 BAEG units/mg), alkaline phosphatase (EC 3.1.3.1) from calf intestine (80 units/mg), collagenase (EC 3.4.24.3) from *Clostridium histolyticum* (830 units/mg), phosphodiesterase (EC 3.1.4.18) from bovine spleen (7.1 units/mg), thrombin from bovine plasma I (600 NIH units/mg), chymotrypsin (EC 3.4.21.1) from bovine pancreas (50 units/mg), β-*N*-acetylglucosaminidase (EC 3.2.1.30) from bovine epididymis (1.5 units/mg), β-glucuronidase (EC 3.2.1.31) from bovine liver (50000 units/mg) and hyaluronate from umbilical cord were obtained from Sigma; Pronase from *Streptomyces griseus* (76600 PUK/g) was from Calbiochem–Behring; hyaluronidase (EC 3.2.1.35) from *Streptomyces hyalurolyticus* (proteinase-free) (2000 NF units/mg) was from Miles; deoxyribonuclease (EC 3.1.21.1) from bovine pancreas (1000 Kunitz units/mg) was from Boehringer; uridinediphospho-D-[U-^{14}C]glucuronic acid (264 mCi/mmol- from Amersham International; uridinediphospho-*N*-acetyl[^{3}H]glucosamine (66 Ci/mmol) was from New England Nuclear.

Oligosaccharides from hyaluronate were obtained by digestion with bovine testis hyaluronidase and chromatography on Sephadex G-50. Hyaluronate standards of defined molecular weight were prepared from a partial digest of umbilical cord hyaluronate with bovine testis hyaluronidase followed by chromatography on Sephacryl S-1000 using a calibration curve kindly provided by Dr. K. Granath.

Cell growth

Teratocarcinoma stem cells F9 were plated in culture flasks at a density of 3×10^4 cells/cm^2 in Dulbecco's modified Eagle medium containing streptomycin–penicillin (100 units/ml) and kana-

Abbreviation used: SDS, sodium dodecyl sulphate.

mycin (100 μg/ml), 10% (v/v) foetal calf serum and 0.1 μM-retinoic acid, which induced differentiation to endodermal-like cells. The mesenchymal-like cell line 756 of teratocarcinoma cells (kindly provided by Dr. R. Kemmler) was grown in the same medium without retinoic acid. Cells were harvested after 3 days by scraping them off or by trypsinization.

Determination of hyaluronate synthesis

The isolated membranes were suspended in 40 mM-sodium phosphate buffer, pH 6.7, at a protein concentration of 5 mg/ml. For hyaluronate synthesis the membranes were incubated in Eppendorf centrifugation vials with the buffer and substrates at final concentrations of 8 μM-UDP-[^{14}C]-GlcA, 166 μM-UDP-GlcNAc, 10 mM-$MgCl_2$, 4 mM-dithiothreitol and various concentrations of ATP or other compounds. Following incubation at 37°C the mixture was centrifuged in an Eppendorf centrifuge 5412 for 3 min at 10000 ***g***. The supernatant and the pellet dissolved in 2% (w/v) SDS were transferred to Whatman 3MM paper and subjected to descending paper chromatography for 16 h with ethanol/aqueous 1 M-ammonium acetate, pH 5.5 (13 : 7, v/v) as solvent. The paper was dried and the radioactivity at the origin was determined.

Inactivation of hyaluronate synthase by proteinases

Membranes (1 mg of protein) were incubated in 70 μl of 40 mM-phosphate buffer, pH 6.4, with 4 μg of the proteinases thrombin, collagenase, trypsin, Pronase or chymotrypsin or in 70 μl of 10 mM-Tris/HCl buffer (pH 7.8)/1.5 mM-$CaCl_2$ with 4 μg of Pronase, trypsin or collagenase, for 15 min at 37°C. The membranes were separated by centrifugation, washed with phosphate buffer and assayed for hyaluronate synthase activity.

Other methods

Protein was determined by the method of Lowry (Markwell *et al.*, 1978). Gel filtration on Sepharose CL 2B was performed on a column (50 cm × 0.8 cm) with phosphate-buffered saline containing 0.1% SDS as eluent; 0.3 ml fractions were collected.

Cells were disrupted by homogenization with 15 strokes of a Potter–Elvehjem B-type pestle, and membranes were prepared by the method of Brunette & Till (1971).

Results

Preparation of hyaluronate synthase

Hyaluronate synthase is induced in teratocarcinoma stem cells F9 by treatment with retinoic acid together with the onset of differentiation to endodermal cells (Prehm, 1980). Extracts of these cells were used in the following experiments.

Hyaluronate synthesis was determined by incubation of the cell extracts with the precursors UDP-GlcNAc and UDP-[^{14}C]GlcA in the presence of $MgCl_2$ and dithiothreitol (Tomida *et al.*, 1975). The hyaluronate synthase was found in the particulate fraction after homogenizing. It resided in the membrane fraction which was obtained from the particulate fraction by the method of Brunette & Till (1971). The synthase activity associated with the membrane fraction could be precipitated by centrifugation at 10000 ***g*** for 3 min in an Eppendorf centrifuge. The separation of the soluble and particulate fraction will serve as the experimental basis for the following kinetic studies on the synthesis and shedding of hyaluronate.

Kinetics of hyaluronate synthesis

The kinetics of hyaluronate synthesis in the membrane preparation was determined by incubation of the membranes with the substrates UDP-GlcNAc and UDP-[^{14}C]GlcA over a period of 5 h. The amount of hyaluronate was measured in the membrane fraction and in the supernatant (Fig. 1). Synthesis ceased after about 60 min, when most of the hyaluronate became soluble.

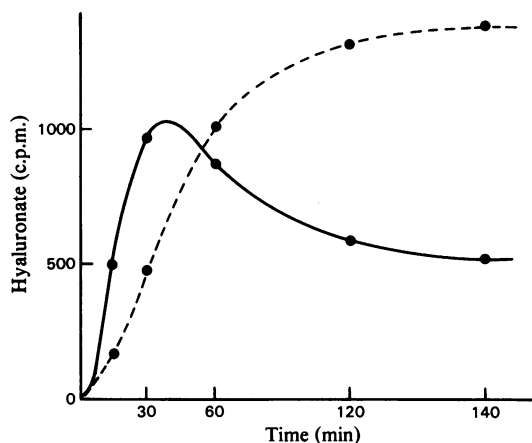

Fig. 1. *Kinetics of hyaluronate synthesis*
A membrane preparation of differentiated teratocarcinoma cells was incubated at a final protein concentration of 2.5 mg/ml with 40 mM-phosphate buffer, pH 6.7, 8 μM-UDP[^{14}C]GlcA (sp. radioactivity 300 μCi/mmol), 166 μM-UDP-GlcNAc, 4 mM-dithiothreitol and 10 mM-$MgCl_2$. At the indicated times 50 μl samples were withdrawn and centrifuged for 3 min at 10000 ***g***. The supernatant and pellet, which was dissolved in 50 μl of 2% SDS, were subjected to paper chromatography. The radioactivity of [^{14}C]hyaluronate at the origin was determined. (——) radioactivity in the pellet; (----) radioactivity in the supernatant.

Stimulation and inhibition of hyaluronate synthesis

ATP has been shown to stimulate hyaluronate synthesis in extracts of mammalian cells at millimolar concentrations (Appel *et al.*, 1979). Fig. 2 shows that hyaluronate is synthesized in the presence of 5mM-ATP for a period of 10h. It also appeared initially to be membrane-bound and was then shed into the supernatant. ATP was believed to protect the substrate from phosphatases (Appel *et al.*, 1979). However, the supernatant from preincubated membranes which had ceased hyaluronate synthesis could still serve as a substrate for hyaluronate synthesis on fresh membranes (Fig. 3). Therefore the substrate was not completely degraded when hyaluronate synthesis had ceased. But also the preincubated membranes themselves were able to resume hyaluronate synthesis with new substrate (Fig. 3). Therefore the stimulating effect of ATP cannot solely be due to the protection of the substrate from degradation.

A number of compounds were screened for their effect on hyaluronic acid synthesis. Fig. 4 shows the concentration-dependent stimulation by various adenosine nucleotides. Adenosine 5′-[γ-thio]triphosphate, adenosine 5′-[β,γ-methylene]triphosphate, adenosine 5′-[β,γ-imido]triphosphate, CoA and GTP influenced hyaluronate synthesis in a similar way as did ATP. But the addition of alkaline phosphatase or phosphodiesterase did not influence hyaluronate synthesis, indicating that the stimulation is specific.

$BeCl_2$ and NH_4VO_3 stimulated the synthesis at a concentration of 10mM.

UTP stimulated the synthesis (Fig. 5), whereas UDP and UMP were inhibitory.

Pyrophosphate, NaF, theophylline, and isopro-

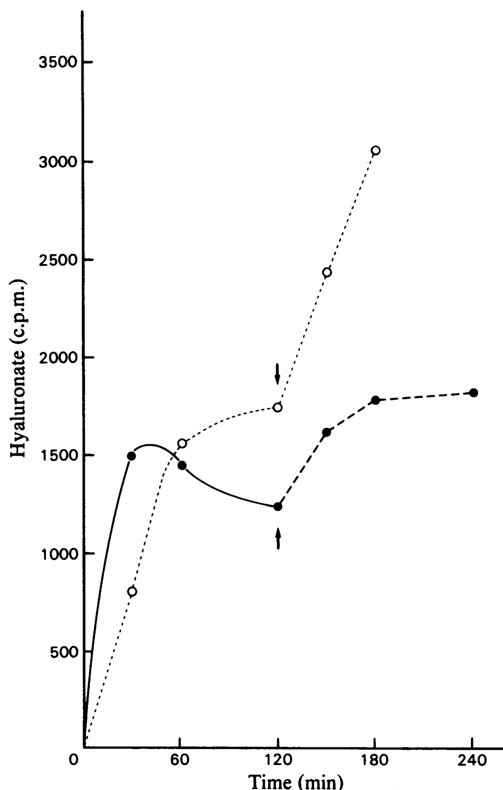

Fig. 3. *Reactivation of hyaluronate synthesis*
Membranes were incubated with substrate in the absence of ATP for 2h and then were isolated by centrifugation (arrows). The supernatant was added to an equivalent amount of fresh membranes (○), and the pellet was suspended in new substrate (●). Equal samples were withdrawn at the time intervals indicated for determination of [^{14}C]hyaluronate.

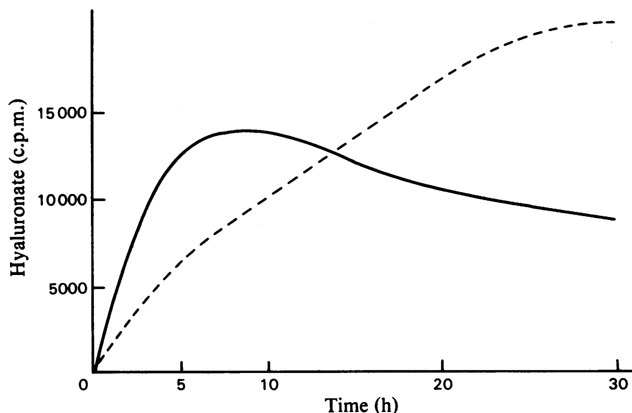

Fig. 2. *Stimulation of hyaluronate synthesis by 5mM-ATP*
The experimental conditions were identical with those described in Fig. 1 except that 5mM-ATP was included in the incubation mixture.

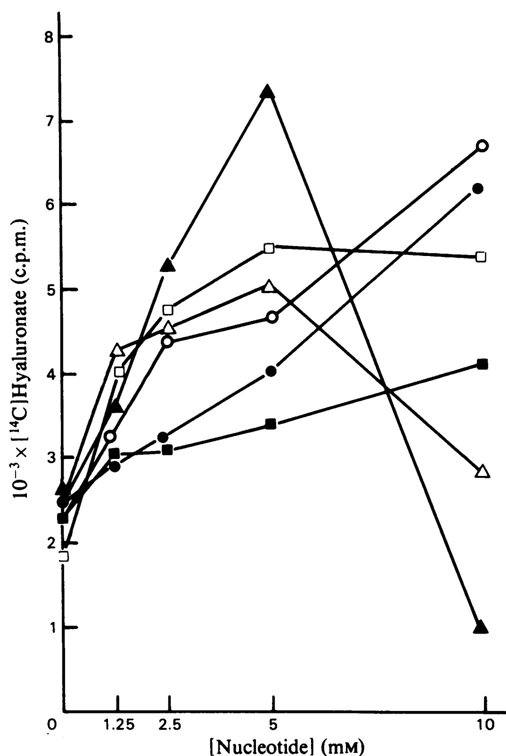

Fig. 4. *Concentration-dependent stimulation of hyaluronate synthesis by adenosine nucleotides*

Membranes (50μl with 150μg of protein) were incubated at 37°C with 8μM-UDP-[^{14}C]GlcA, 166μM-UDP-GlcNAc, 4mM-dithiothreitol, 10mM-$MgCl_2$ and increasing concentrations of adenosine nucleotides for 8h. The reaction was stopped by addition of 5μl of 10% (w/v) SDS and the amount of [^{14}C]hyaluronic acid was determined. ○, ADP; ●, AMP; □, ADP-Glc; ■, cyclic AMP; △, NAD; ▲, ATP.

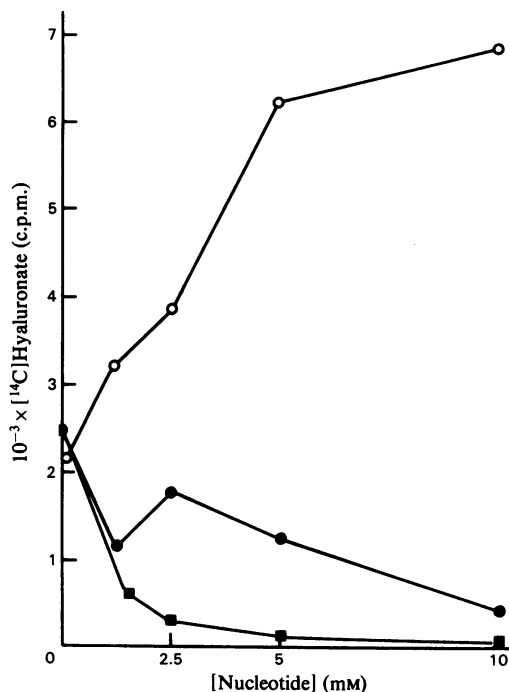

Fig. 5. *Concentration dependency of hyaluronate synthesis in the presence of uridine phosphates*

For conditions of incubation see Fig. 4. ○, UTP; ●, UDP; ■, UMP.

terenol did not influence the synthesis up to a concentration of 10mM.

Detergents and thiol reagents such as iodoacetamide or $HgCl_2$ stopped the synthesis immediately.

Among the proteolytic enzymes tested (thrombin, collagenase, trypsin, Pronase and chymotrypsin) only preincubation with trypsin inhibited the enzyme activity.

The rate of hyaluronate synthesis was not influenced by hyaluronic acid (1mg/ml), by a hexasaccharide from hyaluronate and by pretreatment of the membranes with mammalian or bacterial hyaluronidases (results not shown).

Inactivation of hyaluronate synthase

When membranes were incubated without UDP-GlcNAc and UDP-GlcA at 37°C, they gradually lost their enzyme activity. The decay in the synthesis shown in Fig. 6. This decay was not prevented by ATP and the synthesis could also not be reactivated by later addition of ATP, indicating that ATP did not protect the synthase itself during stimulation of hyaluronate synthesis. The effect of ATP is restricted to the phase of active hyaluronate synthesis, where it maintains a high polymerization rate, even under decreasing substrate concentrations, as shown before.

Release of hyaluronate from the membranes

Membranes were pulse-labelled by incubation with UDP-GlcNAc and UDP-[^{14}C]GlcA for 3h. The membrane-bound [^{14}C]hyaluronate was then further incubated with increasing concentrations of ATP or other phosphate esters and unlabelled UDP-GlcA. After 2.5h of chase the [^{14}C]hyaluronate was determined in the supernatant of the membranes. Fig. 7 shows that increasing concentrations of ATP indeed inhibited the release of hyaluronate from the membranes. Other phosphate esters had the same effect.

When one obligatory component, i.e. $MgCl_2$, UDP-GlcNAc or UDP-GlcA was omitted during the chase period, a low but distinct shedding of the

pulse-labelled hyaluronate was observed which was not influenced by ATP (Fig. 7). Thus ATP prevented shedding only in the active phase of hyaluronate synthesis.

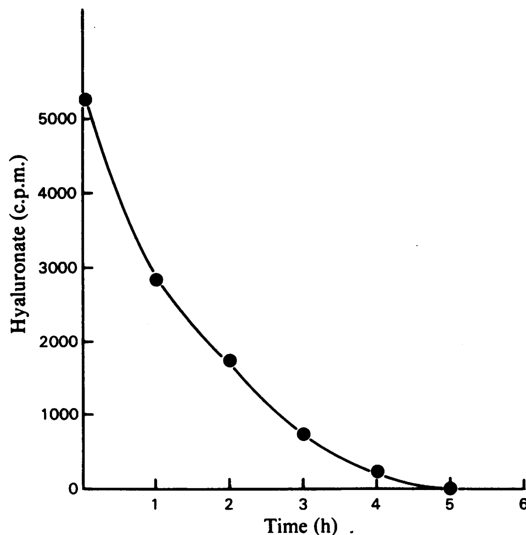

Fig. 6. *Inactivation of hyaluronate synthase*
Membranes were incubated for the times indicated in phosphate buffer, pH 6.7. Then substrate containing 5mM-ATP was added and the enzyme activity was determined after incubation for 1 h.

Table 1. *Release of membrane-bound hyaluronate*
Membranes (50 μg) were labelled by incubation with UDP-[14C]GlcA, UDP-GlcNAc and $MgCl_2$ in phosphate buffer, pH 6.7, for 2h and then separated from the soluble hyaluronate by centrifugation for 2 min at 10000 *g*. After suspension in 50 μl of phosphate buffer the following components were added and incubated for 10 min at 37°C. After centrifugation for 2 min at 10000 *g* the radioactivity of hyaluronate in the pellet and the supernatant was determined. The degree of shedding is expressed as % of hyaluronate released.

Added component	Shedding (%)
Phosphate buffer (40 μM)	21
Triton X-100 (1%)	96
Trypsin (200 μg)	51
UMP (10mM)	25
UDP (10mM)	23
UDP-GlcA (10mM)	29
UDP-GlcNAc (10mM)	34
ATP (10mM)	22
Bacterial hyaluronidase (1 unit)	71
Mammalian hyaluronidase (1 unit)	69
Hyaluronate (1 mg/ml)	23
Hyaluronate hexasaccharide (1 mg/ml)	26

The release of membrane-bound hyaluronate was not influenced by high concentrations of the substrates, by hyaluronate or by a hexasaccharide from hyaluronate (Table 1). The anchorage is therefore specific and hyaluronate cannot be displaced by these compounds.

Detergent released the membrane-bound hyaluronate by solubilization. Trypsin, which had been shown to inhibit the synthesis, could cleave hyaluronate from membranes. Treatment of pulse-labelled hyaluronate with mammalian or bacterial hyaluronidase removed partly the membrane bound chains, but did not interfere with subsequent synthesis.

Kinetics of the hyaluronate chain growth

The chemical identification of the site of hyaluronic acid binding to the membrane requires information about the growth rate of the hyal-

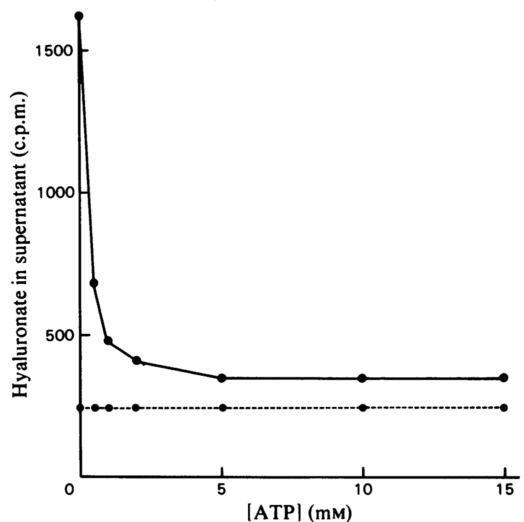

Fig. 7. *Release of pulse-labelled hyaluronate from the membranes in the presence of increasing ATP concentrations*
Membranes (5 mg) were incubated in 1 ml of 40mM-phosphate buffer, pH 6.7, containing 5mM-ATP, 166 μM-UDP-GlcNAc, 8 μM-UDP-[14C]GlcA and 10mM-$MgCl_2$ for 3h. The membranes were separated from the substrate by centrifugation at 10000 *g* for 3 min, washed with phosphate buffer, resuspended in 0.5 ml of phosphate buffer, and 50 μl aliquots of this suspension were mixed with double-concentrated unlabelled substrate solution containing increasing amounts of ATP. The final ATP concentration is indicated on the abscissa. After 2.5h the membranes were again pelleted and the amount of [14C]hyaluronate in the supernatant was determined (——). In another experiment the unlabelled substrate was substituted during the chase period by phosphate buffer (----).

uronate chains and their molar quantity in the membrane extract.

The growth of the hyaluronate chains during incubation of the membranes with radioactive substrate was determined by gel filtration of the product on Sepharose CL2B. This yielded a rough estimate of the molecular weight of the growing hyaluronate chain.

Fig. 8 shows the elution profile from a Sepharose CL2B column of [^{14}C]hyaluronate solubilized by 1% SDS after 15 or 120min incubation of membranes. After 120min of incubation most of the hyaluronic acid is too large to be included by the column. However, there is some tailing of hyaluronate into the included volume of the column which may represent smaller chains. After an incubation of 15min a fraction was excluded from the column. The excluded fraction may represent elongated chains extracted from the membranes and the retarded fraction may be newly synthesized hyaluronate. The sizes of the membrane-bound and released hyaluronic acid showed no differences as shown by their elution profiles on Sepharose CL2B (results not shown). This indicates that hyaluronate is shed randomly independent of its size. Preincubation of the membranes with hyaluronidase from bovine testis eliminated the larger hyaluronate fraction. Fig. 9 shows the elution profile of membranes pretreated with hyaluronidase and then incubated with radioactive substrate for 5, 10, and 15min. The column had been calibrated with hyaluronate standards of defined molecular weight. The elution positions of the peak maxima corresponded to $K_{av.}$ values of 0.51, 0.40 and 0.29 which correlated with molecular weights of 80000, 170000 and 300000, respectively. This growth rate corresponds to 60 GlcNAc-GlcA disaccharide units/min. Assuming that the specific radioactivities of the precursor UDP-[^{14}C]GlcA and the product [^{14}C]-hyaluronate were equal, the amount was calculated

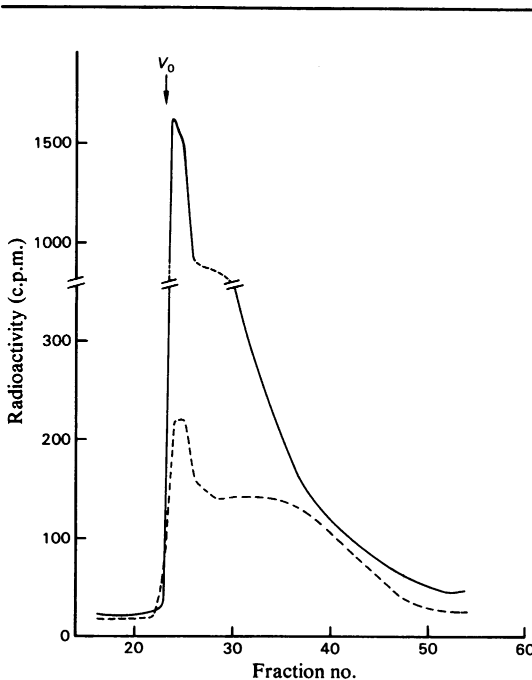

Fig. 8. *Gel chromatography of [^{14}C]hyaluronate on Sepharose CL2B*

Membrane preparation (100μl) was incubated at a protein concentration of 310μg/ml with the hyaluronate-synthesizing substrate solution containing 5mM-ATP for 2h and for 15min. The reaction was terminated by the addition of 10μl of 20% (w/v) SDS and the solution was chromatographed on a Sepharose CL2B column (50cm × 0.65cm) with phosphate-buffered saline containing 0.1% SDS as solvent. Fractions (0.3ml) were collected, and their radioactivity was determined. Elution profiles after 2h (——) and after 15min (----) are shown. The precursor nucleotides were eluted in fractions 57–61 (not shown for simplicity).

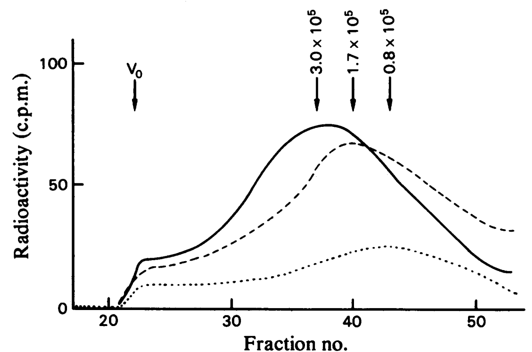

Fig. 9. *Gel chromatography of [^{14}C]hyaluronate on Sepharose CL2B*

A membrane preparation (330μl containing 1.2mg of protein) was incubated with 60NF units of hyaluronidase from bovine testis in 30mM-sodium acetate buffer, pH5.3, for 30min at 37°C. The membrane fraction was sedimented in an Eppendorf centrifuge at 10000g for 5min and washed three times with cold 40mM-phosphate buffer, pH6.7. The sediment was suspended in 300μl of hyaluronate-synthesizing substrate solution containing 5mM-ATP and incubated for 5, 10, and 15min. At these times aliquots (100μl) were withdrawn and the reaction was stopped by addition of 5μl of 20% (w/v) SDS. The mixture was chromatographed on Sepharose CL 2B as described in Fig. 8. Elution profile after 5min (·····), 10min (----), and 15min (——), are shown as well as the molecular weight of the peaks calculated from the elution positions of molecular weight standards.

to be 5.6×10^{-15} mol of hyaluronate/mg of membrane protein.

Chain initiation

Membranes were incubated with 10mM-$MgCl_2$, 5mM-ATP, 10μM-UDP-[^{3}H]GlcNAc and 10μM-UDP-GlcA in 40mM-phosphate buffer, pH 6.7, for 2h and then were chromatographed on Sepharose CL2B. The [^{3}H]hyaluronate consisted of the excluded fraction and some included material eluting as a tail from the Sepharose CL2B column (results not shown). After washing, the membranes were incubated with 10mM-$MgCl_2$, 5mM-ATP, 80μM-UDP-GlcNAc and 3μM-UDP-[^{14}C]GlcA in phosphate buffer for 15 min. The material was rechromatographed on the Sepharose CL2B column, (Fig. 10). The elution profile shows that after the chase with UDP-[^{14}C]GlcA, [^{3}H]hyaluronate eluted as a sharp peak without tailing in the excluded volume of the column. The [^{14}C]hyaluronate appeared in the excluded volume as well as in the included fractions.

This demonstrates that the previously included [^{3}H]hyaluronate had been elongated by UDP-[^{14}C]-GlcA and that the newly synthesized included fraction which contained [^{14}C]hyaluronate must have arisen from synthesis *de novo*. Thus it seemed that the hyaluronate synthase could initiate new chains even after prolonged incubation.

As mentioned above, inclusion of a hexasaccharide from hyaluronic acid or unlabelled hyaluronate did not influence the amount of newly synthesized hyaluronate. If chains were growing at the non-reducing end, the elongation rate should be altered by addition of the hexasaccharide. However, the rate of chain growth was not altered by the hexasaccharide at a concentration of 1 mg/ml as measured by gel filtration on Sepharose CL2B (results not shown). Therefore the non-reducing end of hyaluronate is not an acceptor.

Direction of chain growth

Hyaluronate was pulse-labelled by incubation with UDP-GlcNAc and UDP-[^{14}C]GlcA and chased

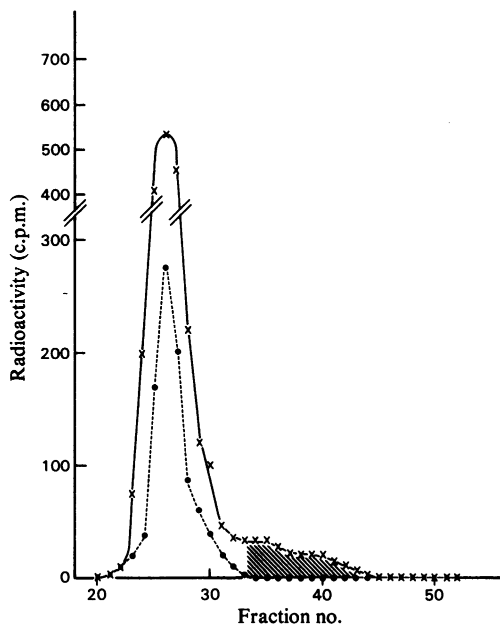

Fig. 10. *Chromatography of pulse–chase double-labelled hyaluronate on Sepharose CL2B*

Membranes were incubated with 10mM-$MgCl_2$, 5mM-ATP, 10μM-UDP-[^{3}H]GlcNAc and 10μM-UDP-GlcA in 40mM-phosphate buffer, pH 6.7, for 2h. After washing they were incubated with 10mM-$MgCl_2$, 5mM-ATP, 80μM-UDP-GlcNAc and 3μM-UDP-[^{14}C]GlcA in phosphate buffer for 15 min. The material was rechromatographed on Sepharose CL2B. ×——×, ^{14}C; ●-- ●, ^{3}H.

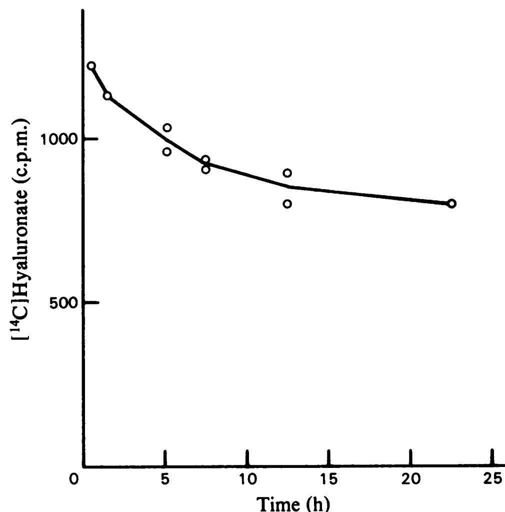

Fig. 11. *Degradation of pulse–chase-labelled hyaluronate by β-N-acetylglucosaminidase and β-glucuronidase*

Membranes (2.6 mg) were incubated in 200μl of phosphate buffer containing 10mM-$MgCl_2$, 5mM-ATP, 4mM-dithiothreitol, 8μM-UDP-[^{14}C]GlcA and 160μM-UDP-GlcNAc for 15 min at 37°C, centrifuged at 100000**g** for 5 min and further incubated in the same buffer with unlabelled UDP-GlcA for 15 min. The membranes were again centrifuged off and suspended in 2.2 ml of 50mM-sodium citrate/50mM-sodium acetate (pH 4.3), containing 1mM-EDTA, 50000 units of β-glucuronidase and 0.5 units of β-*N*-acetylglucosaminidase in dialysis bags. During dialysis against the above citrate/acetate buffer, 150μl samples were withdrawn and subjected to paper chromatography. [^{14}C]Hyaluronate was determined as described in the Materials and methods section.

with unlabelled UDP-GlcA. It was then incubated with β-*N*-acetylglucosaminidase and β-glucuronidase (Longas & Meyer, 1981), in order to determine the site of the incorporated radioactivity in the pulse–chase-labelled chains. The products of enzyme digestion were dialysed out during incubation and the remaining [^{14}C]hyaluronate was determined (Fig. 11).

The immediate drop in [^{14}C]hyaluronate proved that the radioactive part of the chain was located at the non-reducing end. Thus chain growth must occur at the reducing end. The residual level of slowly digestible [^{14}C]hyaluronate probably represented chains which were present in the membrane preparation and elongated at the reducing end. Degradation by lysosomal enzymes can be excluded, because incubation in the absence of added enzymes did not reduce the amount of hyaluronate. Hyaluronidase contamination of the enzyme preparation is unlikely, because it would degrade the pulse–chase-labelled chains completely and not stop at the residual level.

Discussion

Hyaluronate was synthesized on membranes and then shed. Under the incubation conditions hyaluronate was the only radioactive component synthesized, as shown by ion exchange chromatography, gel filtration of the product on Sepharose CL2B, digestion of the high-molecular-weight fraction with *Streptomyces* hyaluronidase, its ability to mediate aggregation of cartilage proteoglycans (Hascall & Heinegard, 1974), and its resistance to deamination by HNO_2 (Lindahl *et al.*, 1973). Particulate and soluble hyaluronate were separated by centrifugation. This method was preferred to precipitation with trichloroacetic acid used previously (Appel *et al.*, 1979), in order to keep the enzyme functional for further experiments.

Hyaluronate synthesis was stimulated by a wide variety of compounds. They resembled each other only in the phosphate ester moiety. $BeCl_2$ and NH_4VO_3 are inhibitors of phosphatases (Fernley, 1971; Macara, 1980). This suggested that these chemicals act by inhibition of phosphatases or 5′-nucleotidase.

Membranes ceased hyaluronate synthesis in the absence of ATP after about 2h. However, they could be reactivated when exposed to the original high concentration of UDP-GlcNAc and UDP-GlcA, and the substrate concentration after 2h was high enough to support synthesis on fresh membranes. Therefore, ATP seemed to stimulate hyaluronate synthesis not only by protecting the substrate from degradation, but also be maintaining the hyaluronate synthase active at decreasing substrate concentrations. Therefore, the growing hyaluronate chains could be anchored at the membranes by a phosphodiester bridge. Release of hyaluronate from membranes could be caused by a phosphodiesterase.

UDP and UMP probably inhibited the synthesis by blocking the active site because of their structural similarity to the substrates UDP-GlcNAc and UDP-GlcA.

Gel filtration of [^{14}C]hyaluronate always showed some tailing in the included volume of the Sepharose CL2B column, even after prolonged incubation. Thus the question arose whether the included fraction could represent growing chains which were synthesized *de novo*, and whether new chain 'starts' could be initiated at any time during incubation. Hyaluronate was first labelled with UDP-[^{3}H]-GlcNAc and chromatographed on Sepharose CL2B. If the first incubation with UDP-[^{3}H]GlcNAc had charged all enzyme sites, initiation of new chains during further incubation would require a release of synthesized chains from the enzyme. This has already been demonstrated (Figs. 1 and 2). The appearance of [^{14}C]hyaluronate in the excluded as well as in the included fractions demonstrated that new chains were started with UDP-[^{14}C]GlcA. Continuous new chain initiation during incubation required therefore an indefinite pool of primer molecules or a mechanism of synthesis which is independent of primers.

Oligosaccharides from hyaluronate were not acceptors for the synthase and hyaluronidase treatment did not influence further synthesis.

Directional degradation of pulse–chase-labelled hyaluronate with β-*N*-acetylglucosaminidase and β-glucuronidase showed that the chains grew at the reducing end.

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