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Analysis of cell-growth-phase-related variations in hyaluronate synthase activity of isolated plasma-membrane fractions of cultured human skin fibroblasts

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Hyaluronate synthase activity is localized exclusively in plasma-membrane fractions of cultured human skin fibroblasts. The enzyme activity of plasma membranes prepared from exponential-growth-phase cells was about 6.5 times that of stationary-growth-phase cells. Hyaluronate synthase from exponential-growth-phase cells exhibited lower K_m and higher $V_{max.}$ values for both UDP-*N*-acetylglucosamine and UDP-glucuronic acid and higher rate of elongation of hyaluronate chains compared with the enzyme from stationary-growth-phase cells. Hyaluronate synthase exhibited an extremely short half-life, 2.2 h and 3.8 h respectively when cells were treated with cycloheximide and actinomycin D. The cell-growth-phase-dependent variations in hyaluronate synthase activity appear to be due to its high turnover rate as well as due to some post-translational modification of the enzyme protein as cells progress from early exponential to stationary growth phase. The isolated plasma membranes contained a protein (M_r approx. 450000) that was selectively autophosphorylated from [γ-^{32}P]ATP *in vitro* in the presence of hyaluronate precursors in the reaction mixture and that also exhibited some hyaluronate-synthesis-related properties. The ^{32}P-labelled protein isolated from plasma membranes of exponentially growing cells expressed an efficient UDP-[^{14}C]glucuronic acid- and UDP-*N*-acetyl[^{3}H]glucosamine-binding activity and was able to synthesize oligosaccharides (M_r 5000) of [^{14}C]glucuronic acid and *N*-acetyl[^{3}H]glucosamine residues. The corresponding protein of stationary-growth-phase cells, which expressed much higher nucleotide-sugar-precursor-binding activity, appeared to have lost its oligosaccharide-synthesizing activity.

INTRODUCTION

Hyaluronate synthesis is modulated during the migration of mesenchymal cells (Toole, 1972), the invasion of carcinoma cells (Toole *et al.*, 1979), virus transformation of fibroblasts (Hopwood & Dorfman, 1977) and differentiation of teratocarcinoma cells (Prehm, 1980). Similarly an enhanced production of hyaluronate is also reported in fibroblast cultures of patients with Marfan's syndrome (an autosomal dominant gene defect) or with cystic fibrosis (an autosomal recessive gene defect) (Appel *et al.*, 1979; Matalon & Dorfman, 1968). Hyaluronate synthesis in normal cultured fibroblasts is also shown to be enhanced by medium change, foetal bovine serum, cyclic AMP and its dibutyryl cyclic AMP analogue, prostaglandin E and Ca^{2+} (Tomida *et al.*, 1975; Koyama *et al.*, 1976; Tajima *et al.*, 1981). However, the mechanism of modulation of hyaluronate synthesis is not clarified as yet.

In normal fibroblasts a decrease in the rate of hyaluronate synthesis has been shown as cultured cells progressed from low to high densities (Tomida *et al.*, 1975; Kittlick *et al.*, 1976*a*,*b*; Hronowski & Anastassiades, 1980; Prehm, 1980) and from exponential to stationary growth phase (Tomida *et al.*, 1974, 1975). A direct and comparative study on the properties of hyaluronate synthase from exponential- and stationary-growth-phase cells has not been carried out hitherto. Although hyaluronate synthase, a putative enzyme system catalysing the polymerization of glucuronic acid and *N*-acetylglucosamine residues, derived from UDP-glucuronic acid and UDP-*N*-acetylglucosamine precursors, into high-M_r (10^6–10^7) hyaluronate chains has not been purified as yet, studies have shown that the hyaluronate-synthesizing activity resides exclusively in the plasma membrane of the cell (Appel *et al.*, 1979; Prehm, 1984; Mian, 1984). The present work was therefore carried out to investigate the mechanism(s) affecting the expression of hyaluronate synthase activity in plasma membranes of normal cultured human skin fibroblasts in exponential and stationary growth phases.

MATERIALS AND METHODS

Fibroblast cultures

Four normal human skin fibroblast cell lines were kindly provided by Dr. Fensom, Guy's Hospital Medical School, London, U.K. Cells were routinely grown in Ham's F-10 medium with 20 mM-Hepes buffer and supplemented with 10% (v/v) foetal bovine serum, 0.075% $NaHCO_3$, 2 mM-glutamine and penicillin (50 units/ml)/streptomycin (50 μg/ml). Cells were harvested by use of a trypsin/EDTA mixture (0.05% trypsin and 0.02% EDTA in a special balanced salt solution) and counted and split into a 1:2 ratio for routine subculturing. For the enzyme studies, cells were removed from the culture flasks by using disposable plastic cell scrapers. All cell-culture material was obtained ready-made from the Flow Laboratories, Rickmansworth, Herts., U.K.

Preparation of plasma-membrane fraction

The plasma-membrane-rich fraction was prepared by the method of Evans (1980). Cells were homogenized in 10% (w/v) sucrose in 10 mM-Tris/HCl buffer, pH 7.1, by using Potter–Elvehjem homogenizer (radial clearance 0.038 mm). The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant separated and re-centrifuged at 10000 *g* for 10 min. The resulting pellet was collected and resuspended in 30% (w/v) sucrose in 10 mM-Tris/HCl buffer, pH 7.1, and applied to a linear 30–50% (w/v) sucrose gradient in the same buffer. The sample was centrifuged at 90000 *g* for 3 h. The plasma-membrane-rich band, at equilibrium density 1.145 g/cm^3, was separated and washed twice with 50 mM-Tris/HCl buffer, pH 7.1, by centrifugation at 10000 *g* for 10 min. The purity of the plasma-membrane fraction was authenticated by comparison of specific markers, such as DNA for cell nuclei, 5′-nucleotidase (EC 3.1.3.5) and alkaline phosphatase (EC 3.1.3.1) for plasma membrane, NADPH–cytochrome *c* reductase (EC 1.6.2.4) for endoplasmic reticulum, succinate dehydrogenase (EC 1.3.99.1) for mitochondria, acid phosphatase (EC 3.1.3.2) and β-glucuronidase (EC 3.2.1.31) for lysosomes, *N*-acetylglucosamine galactosyltransferase (EC 2.4.1.38) for Golgi membranes and lactate dehydrogenase (EC 1.1.1.22) for cytoplasm, in the plasma-membrane fraction with those of whole-cell homogenate. By these criteria, the plasma-membrane fraction contained about 80% and 60% of the total 5′-nucleotidase and alkaline phosphatase activities but was extremely poor (less than 2% of the total) in other enzymic activities. Besides the absence of any detectable activity of *N*-acetylglucosamine galactosyltransferase (a Golgi-membrane marker), plasma membranes did not show any glucuronosyltransferase or *N*-acetylglucosaminosyltransferase activities other than that of hyaluronate synthesis activity. The plasma-membrane fraction represented only 2% of the total homogenate protein.

Protein and enzyme activity determinations

Protein (Lowry *et al.*, 1951), DNA (Burton, 1956) and activities of 5′-nucleotidase (Ipata, 1968), alkaline phosphatase (Pekarthy *et al.*, 1972), NADPH–cytochrome *c* reductase (Sottocasa *et al.*, 1967), succinate dehydrogenase (Earl & Korner, 1965), acid phosphatase and β-glucuronidase (Gianetto & de Duve, 1955), *N*-acetylglucosamine galactosyltransferase (Bergeron *et al.*, 1973) and lactate dehydrogenase (enzyme-test combination kit, Boehringer) were determined according to the methods in the references cited in parentheses.

Gel chromatography

Gel chromatography of the samples was carried out on Sepharose CL-4B (Pharmacia, Hounslow, Middx., U.K.) and Bio-Gel P-6 (Bio-Rad Laboratories, Watford, Herts., U.K.), depending on the nature of the experiment. Sepharose CL-4B columns were calibrated with the standard proteins α-lactalbumin (M_r 14200), carbonic anhydrase (M_r 29000), chicken egg albumin (M_r 45000), bovine serum albumin (M_r 66000), *Escherichia coli* galactosidase (M_r 116000), rabbit muscle myosin (M_r 205000) and apoferritin monomer (M_r 450000), all obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Determination of hyaluronate synthase activity

The hyaluronate synthase activity was determined according to the methods of Tomida *et al.* (1974) and Appel *et al.* (1979), with some modification. The final reaction mixture (40 μl) contained 50 mM-Hepes/NaOH buffer, pH 7.1, 5 mM-$MgCl_2$, 10 μM-UDP-*N*-acetylglucosamine, 10 μM-UDP-glucuronic acid, 5 mM-ATP and approx. 100 μg of plasma-membrane protein, unless otherwise stated. Depending on the nature of the experiment, either UDP-D-[U-^{14}C]glucuronic acid or UDP-*N*-acetyl-D-[6-^{3}H]glucosamine or both radiolabelled nucleotide sugars were used. In some experiments the enzyme reaction mixture was supplemented with other test reagents, and in the case of reaction kinetic experiments concentrations of UDP-*N*-acetylglucosamine and UDP-glucuronic acid were varied. Following incubation, 10 μl of 10% SDS was added and samples were boiled for 2 min at 100 °C. The samples were then applied to Whatman 3MM paper and subjected to descending chromatography in isobutyric acid/1 M-NH_3 (5:3, v/v). After 48 h development the spotted origins of the chromatogram were cut out and radioactivity was measured.

Radiochemicals and other reagents

UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (> 200 mCi/mmol), UDP-D-[U-^{14}C]glucuronic acid (> 225 mCi/mmol), UDP-D-[U-^{14}C]galactose (> 200 mCi/mmol), UDP-*N*-acetyl-D-[6-^{3}H]glucosamine (5–25 Ci/mmol) and [γ-^{32}P]ATP (> 10 Ci/mmol) were purchased from Amersham International, Amersham, Bucks., U.K., and from New England Nuclear, Du Pont, Southampton, U.K. Hyaluronidase (EC 3.2.1.35) from bovine testes (15000 NF units/mg), β-*N*-acetylglucosaminidase (EC 3.2.1.30) from bovine kidney (15 units/mg), β-glucuronidase (EC 3.2.1.31) from bovine liver (50000 units/mg) and hyaluronate from umbilical cord were obtained from Sigma Chemical Co. All appropriate substrates for assaying marker enzymes during purification of plasma-mebrane fractions were purchased from Sigma Chemical Co. All other chemicals, AnalaR or laboratory-grade reagents, were obtained from BDH Chemicals, Poole, Dorset, U.K., or Sigma Chemical Co.

RESULTS

Cell-growth-phase-related variations in hyaluronate synthase activity

Preliminary experiments with 10000 *g* particulate fraction of cell homogenates prepared from 24 h-, 48 h-, 72 h-and 96 h-old cultured cells showed considerable variation in their particulate-bound enzyme activity (Fig. 1). The enzyme activity increased rapidly during early exponential growth phase (0–24 h period) with cell density increasing from approx. 3×10^3 cells/cm^2 to 9×10^3 cells/cm^2. The rate of increase of enzyme activity then declined slightly during the next 24 h period of growth, with cell density reaching approx. 23×10^3 cells/cm^2. During the next 48 h period of growth, with cells entering stationary growth phase and cell density reaching approx. 35×10^3 cells/cm^2, the enzyme activity also started to decline rapidly (Fig. 1).

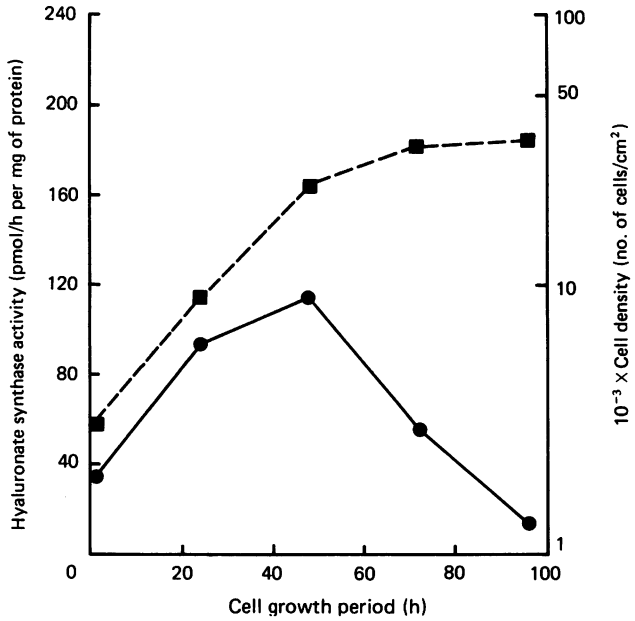

Fig. 1. Cell-growth-phase-related changes in hyaluronate synthase activity

Cells (5×10^5), harvested from confluent monolayers, were cultured in 20 ml of Ham's F-10 medium containing 10% foetal bovine serum in 175 cm^2 flasks. Fifteen flasks were harvested daily, when cells were removed from three representative flasks by trypsinization for counting and from other twelve flasks by scraping for enzyme studies. The cells were homogenized in 10% (w/v) sucrose in 10 mM-Tris/HCl buffer, pH 7.1, and centrifuged at 1000 *g* for 10 min. The resulting supernatant was then centrifuged at 10000 *g* for 10 min, and the pellet collected. The pellet was washed twice with 50 mM-Tris/HCl buffer, pH 7.1, before determination of its hyaluronate synthase activity as described in the Materials and methods section. The results given are average values for three experiments. ●, Hyaluronate synthase activity; ■, cell density.

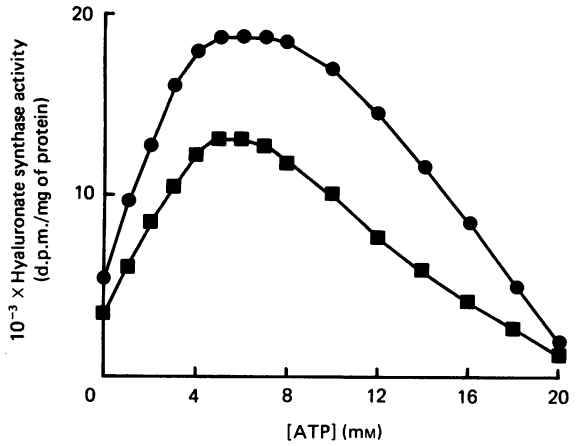

Fig. 2. Effect of ATP on hyaluronate synthase activity

The plasma-membrane samples (approx. 200 μg) were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-Mg^{2+}, 10 μM-UDP-glucuronic acid, 10 μM-UDP-*N*-acetylglucosamine and 0.01 μCi of UDP-*N*-acetyl[^{3}H]-glucosamine. The concentration of ATP in the reaction mixture was varied over the range 0–20 mM. The incubation was carried out at 37 °C for 1 h. The values plotted are the average for three experiments. ●, Plasma membranes of 24 h-old cells; ■, plasma membranes of 72 h-old cells.

Preparation of plasma-membrane-bound hyaluronate synthase

Almost all recoverable hyaluronate synthase activity (98%) was present in the 10000 *g* pellet of the cell homogenate. The purification of plasma-membrane-rich fraction from the 10000 *g* pellet enriched the enzyme activity by 20–24-fold. The resultant activities of enzyme from 24 h-, 48 h-, 72 h- and 96 h-old cell preparations (means ± S.E.M.) were 22.22 ± 0.16 ($n = 18$), 2.60 ± 0.24 ($n = 3$), 1.06 ± 0.12 ($n = 10$) and 0.34 ± 0.04 ($n = 15$) nmol/h per mg of protein respectively.

General properties of plasma-membrane-bound hyaluronate synthase

The enzyme activity was linear with increasing amount of membrane protein up to 240 μg/reaction mixture and up to 45 and 30 min with plasma membranes of 24 h- or 48 h-old cells and of 72 h- or 96 h-old cells respectively. Maximal enzyme activity was observed at pH 7.0–7.1 in buffer systems such as 50 mM-Na_2HPO_4/NaH_2PO_4, 50 mM-Tris/HCl or 50 mM-Hepes/NaOH. Whereas the presence of Mg^{2+} (5 mM) was essential for the expression of maximal enzyme activity, other bivalent cations such as Ca^{2+} or Mn^{2+} (10 mM) had no effect.

In the absence of ATP from the reaction mixture, the plasma membrane expressed only a much diminished basal enzyme activity (Fig. 2). Maximal stimulation was, however, observed at 5–7 mM-ATP, and a further increase in ATP was found to inhibit the enzyme activity (Fig. 2). The addition of ATP (5 mM) also decreased the breakdown of UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine from 55% and 95% respectively to about 20% in both cases. The stimulatory action of ATP did not appear to be due to its role in preventing hydrolysis of nucleotide sugar substrates, since in the presence of excess of substrates (at 100 μM), sufficient to overcome their depletion due to non-specific hydrolysis, the enzyme activity did not rise above its base-line level in the absence of ATP (Table 1). The addition of ATP (5 mM), which increased enzyme activity by more than 10-fold, confirmed that its presence was essential for the stimulation of enzyme activity and that high concentrations of nucleotide sugar substrates did not inhibit the enzyme (Table 1). ATP did not affect hyaluronate synthase activity by protecting the enzyme protein from decay, since storage of plasma membranes in buffer solutions with or without ATP had no effect on their enzyme activity as long as enzyme assays were performed in the presence of ATP in the reaction mixture. The effects of other nucleotides and reagents on hyaluronate synthase activity of plasma membranes prepared from 24 h- and 96 h-old cells are shown in Table 2.

Reaction constants of plasma-membrane hyaluronate synthase

The K_m values (means ± S.E.M.) for UDP-glucuronic acid and UDP-*N*-acetylglucosamine (1.60 ± 0.32 and 12.2 ± 1.85 μM, $n = 7$) of enzyme from 24 h-old cells were lower than corresponding vaues (3.6 ± 0.75 and 22.4 ± 2.15 μM, $n = 5$) of enzyme from 96 h-old cells. On the other hand, $V_{max.}$ values (means ± S.E.M.) for

Table 1. Stimulation of plasma-membrane bound hyaluronate synthase activity by ATP

The plasma-membrane fractions (100–200 μg) were incubated, in triplicate, in reaction mixtures as listed below. The reaction mixtures were made up in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-$MgCl_2$. The samples were incubated at 37 °C for 30 min and hyaluronate synthase activity was determined as described in the Materials and methods section. The values given are means ± S.E.M. for seven experiments.

	Plasma-membrane-bound hyaluronate synthase specific activity			
	24 h-old cells		96 h-old cells	
Reaction mixture	(nmol/h per mg)	(%)	(nmol/h per mg)	(%)
10 μM-UDP-GlcA + 10 μM-UDP-GlcNAc + 5 mM-ATP	2.25 ± 0.16	100	0.32 ± 0.02	100
10 μM-UDP-GlcA + 10 μM-UDP-GlcNAc	0.21 ± 0.03	9.3	0.02 ± 0.01	6.2
100 μM-UDP-GlcA + 100 μM-UDP-GlcNAc + 5 mM-ATP	2.36 ± 0.11	104.8	0.38 ± 0.04	118.7
100 μM-UDP-GlcA + 100 μM-UDP-GlcNAc	0.23 ± 0.2	10.2	0.02 ± 0.01	6.2

Table 2. Effect of various agents on plasma-membrane-bound hyaluronate synthase activity

The plasma-membrane samples (50–100 μg) were incubated, in triplicate, in control and experimental reaction mixtures made up by adding or omitting the agents listed below. The hyaluronate synthase activity was determined after 30 min incubation at 37 °C. The values given are means ± S.E.M. for five separate experiments.

	Plasma membrane-bound hyaluronate synthase specific activity			
	24 h-old cells		96 h-old cells	
Agents added or omitted	(nmol/h per mg)	(%)	(nmol/h per mg)	(%)
None (control)	2.16 ± 0.13	100	0.29 ± 0.04	100
+ ADP (5 mM)	1.78 ± 0.16	82.4	0.24 ± 0.03	82.7
− ATP + ADP (5 mM)	0.21 ± 0.03	9.7	0.03 ± 0.01	10.3
+ AMP (5 mM)	2.11 ± 0.17	97.7	0.28 ± 0.03	96.5
− ATP + AMP (5 mM)	0.19 ± 0.06	8.8	0.03 ± 0.01	10.3
+ GTP (5 mM)	2.16 ± 0.16	100	0.28 ± 0.04	96.5
− ATP + GTP (5 mM)	0.15 ± 0.05	6.9	0.03 ± 0.01	10.3
+ UTP (5 mM)	2.37 ± 0.31	109.7	0.26 ± 0.06	89.6
− ATP + UTP (5 mM)	0.18 ± 0.06	8.3	0.03 ± 0.02	10.3
$-Mg^{2+}$	0.05 ± 0.03	2.3	0.01 ± 0.01	3.4
$-Mg^{2+} + Ca^{2+}$ (5 mM)	0.04 ± 0.03	1.8	0.01 ± 0.01	3.4
$+Ca^{2+}$ (5 mM)	2.12 ± 0.02	98.1	0.26 ± 0.06	89.6
+ Cyclic AMP (100 μM)	2.15 ± 0.22	99.5	0.25 ± 0.05	86.2
+ Cyclic GMP (100 μM)	2.11 ± 0.16	97.6	0.22 ± 0.06	75.8
+ Calmodulin (100 μg/ml)	2.12 ± 0.31	98.1	0.24 ± 0.05	82.8
+ Calmodulin (100 μg/ml) + Ca^{2+} (5 mM)	2.13 ± 0.16	98.6	0.27 ± 0.06	93.1
+ GlcA (100 μM)	1.78 ± 0.24	82.4	0.21 ± 0.04	72.4
+ GlcNAc (100 μM)	2.56 ± 0.32	118.5	0.36 ± 0.08	124.1
+ Lysophosphatidylcholine (4 μg/ml)	2.06 ± 0.17	95.3	0.28 ± 0.04	96.5
+ Lysophosphatidylethanolamine (4 μg/ml)	2.15 ± 0.21	99.5	0.23 ± 0.05	79.3
+ Lysophosphatidylcholine (4 μg/ml + calmodulin (100 μg/ml) + Ca^{2+} (5 mM) + cyclic AMP (100 μM)	2.09 ± 0.18	96.7	0.24 ± 0.06	82.7
+ Foetal bovine serum (10%)	2.17 ± 0.20	100.5	0.29 ± 0.05	100

UDP-glucuronic acid and UDP-*N*-acetylglucosamine (6.88 ± 0.75 and 7.65 ± 0.64 nmol/h per mg, $n = 7$) of enzyme from 24 h-old cells were higher than corresponding values (0.75 ± 0.12 and 0.82 ± 16 nmol/h per mg, $n = 5$) of enzyme from 96 h-old cells. The lower K_m and higher $V_{max.}$ values for hyaluronate synthase preparations from 24 h-old cells may explain the higher hyaluronate-synthesizing activity of these cells compared with those of 96 h-old cells. As reported by previous workers (Stoolmiller & Dorfman, 1969; Appel *et al.*, 1979), the K_m for UDP-*N*-acetylglucosamine was approx. 6-fold that for UDP-glucuronic acid.

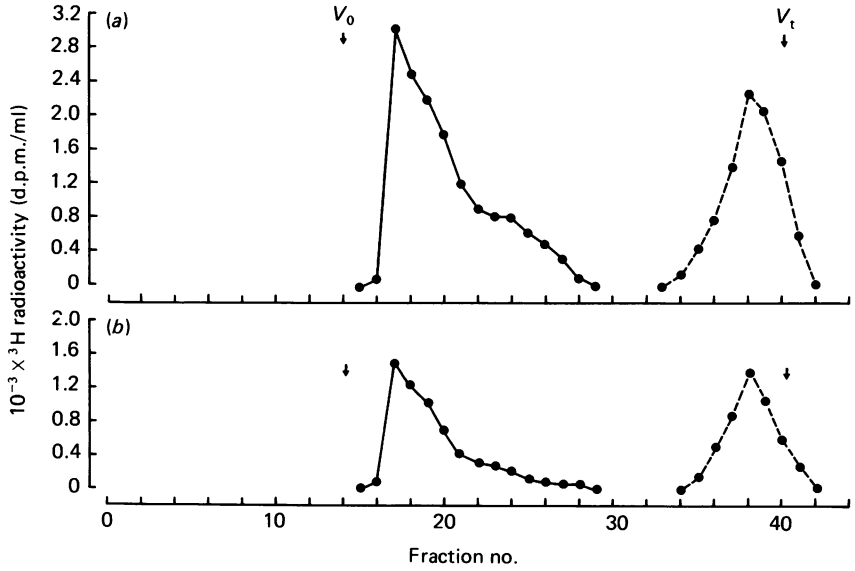

Fig. 3. Analysis of hyaluronate produced by hyaluronate synthase reaction

The plasma membrane samples (approx. 200 μg) were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-ATP, 5 mM-Mg^{2+}, 100 μM-UDP-glucuronic acid, 100 μM-UDP-*N*-acetylglucosamine and 0.05 μCi of UDP-*N*-acetyl [^{3}H]glucosamine for 1 h at 37 °C. The reaction was stopped by heating samples at 100 °C for 2 min and the samples were subjected to gel filtration on Sepharose CL-4B as described in the Materials and methods section; ●—● represents elution patterns of hyaluronate synthesized by plasma membrane prepared from 24 h- and 72 h-old cells. Fractions 16–26 from both columns were pooled separately, concentrated, dialysed against 20 mM-sodium acetate buffer, pH 5.5 and incubated with hyaluronidase (500 NF units) for 6 h at 37 °C. The reaction mixture was rechromatographed on the same columns; ●--● represents elution patterns after hyaluronidase digestion. (*a*) Plasma-membranes of 24 h-old cells; (*b*) plasma membranes of 72 h-old cells. V_0, void volume; V_t, total volume.

Analysis of reaction products of hyaluronate synthase

The gel-filtration profiles of radioactivity of reaction mixtures, showing a sharp peak near the void volume and some tailing into the included volume of Sepharose CL-4B columns (Fig. 3), suggest the presence of high-M_r hyaluronate ($M_r > 2 \times 10^6$), too large to be included by the column, and of smaller chains of hyaluronate. When both high-M_r and low-M_r materials were treated with hyaluronidase and rechromatographed, all radioactivity was recovered in low-M_r fragments (Fig. 3), indicating that the initial product of enzyme reaction was hyaluronate. Fig. 3 also shows no significant difference in the M_r profiles of hyaluronate molecules synthesized by plasma membranes of 24 h-and 72 h-old cells during a 1 h incubation.

Rate of hyaluronate chain elongation

The rate of elongation of hyaluronate molecules synthesized by plasma-membrane-bound enzyme was determined by gel filtration of reaction products after 1, 5 and 10 min of incubation. The elution positions of the peak maxima corresponded to approx. M_r 200000, 120000 and 20000 for reaction products of plasma membranes of 24 h-old cells and to approx. M_r 150000, 60000 and 16000 for reaction products of plasma membranes of 72 h-old cells (Fig. 4). From these approximate M_r estimations the rate of hyaluronate chain elongation by hyaluronate synthase of 24 h- and 72 h-old cells corresponds to about 50–60 and 30–40 disaccharide units/min respectively.

Hyaluronate chain initiation and termination

Hyaluronate chains during their synthesis are not covalently linked to plasma-membrane-bound enzyme, nor does the UDP moiety of nucleotide sugar molecules on the growing end of hyaluronate chain form a link between the enzyme and hyaluronate molecule as suggested by Prehm (1983). The removal of UDP-glucuronic acid and UDP-*N*-acetylglucosamine and washing of membranes with 50 mM-Tris/HCl buffer, pH 7.1, or 0.5% (w/v) SDS or precipitation with 5% (w/v) trichloroacetic acid completely removed all hyaluronate molecules from the plasma-membrane fraction. Experiments with [^{3}H]UDP-glucuronic acid or [^{3}H]UDP-*N*-acetylglucosamine (Mian, 1986) as hyaluronate precursors showed no evidence of ^{3}H radioactivity being incorporated into the plasma-membrane fraction or newly synthesized hyaluronate chains. Re-incubation of membrane samples washed with 50 mM-Tris/HCl buffer, pH 7.1, under hyaluronate-synthesis reaction conditions re-initiated synthesis of new hyaluronate chains. Since the chain initiation appears to start at the beginning, the synthesis of hyaluronate chains of different M_r during a fixed incubation period, as shown in Fig. 3, suggests that both elongation and termination of chain growth depend on the turnover rate of hyaluronate-synthesis complex in the membrane preparations.

Turnover rate of plasma-membrane-bound hyaluronate synthase in the cell

The turnover rate of plasma membrane-bound hyaluronate synthase was determined by the method

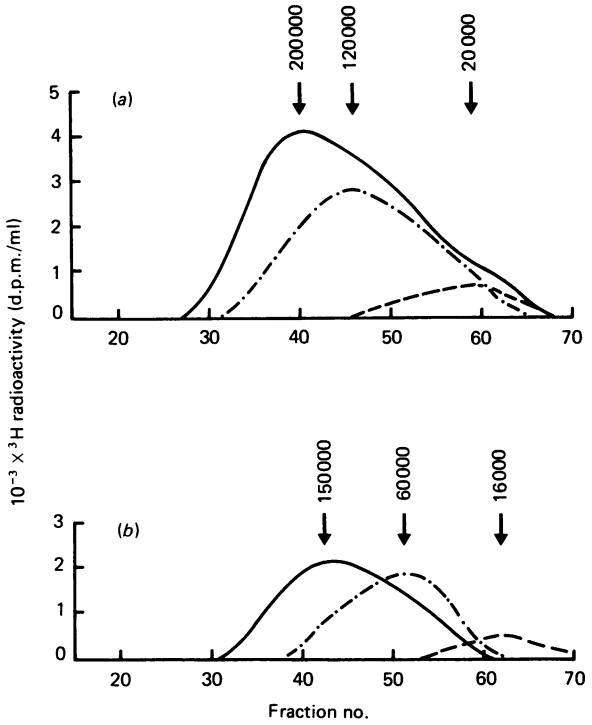

Fig. 4. Rate of elongation of hyaluronate molecules

The plasma-membrane samples (approx. 250 μg) of 24 h- and 72 h-old cells were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-Mg^{2+}, 5 mM-ATP, 100 μM-UDP-glucuronic acid, 100 μM-UDP-*N*-acetylglucosamine and 0.05 μCi of UDP-*N*-acetyl[^{3}H]glucosamine at 37 °C for 1, 5 and 10 min. The reaction was stopped by heating samples at 100 °C for 2 min, and samples were subjected to gel filtration on Sepharose CL-4B columns. The columns were precalibrated with standard M_r protein markers and a commercial preparation of hyaluronate (M_r 400000; Sigma Chemical Co.) and its hyaluronidase digests. The peak maximum of the test sample was compared with those of the M_r standards. Elution patterns of hyaluronate molecules synthesized after 10 min (——), 5 min (— · —) and 1 min (— — —) incubation period are shown. (*a*) Plasma membranes of 24 h-old cells; (*b*) plasma membranes of 72 h-old cells.

described by Tomida *et al.* (1974). The plasma-membrane-bound hyaluronate synthase activity of 24 h-old cells started to decline shortly after the treatment with metabolic inhibitors, exhibiting a half-life of 2.2 h in the presence of cycloheximide and 3.8 h in the presence of actinomycin D (Fig. 5*a*). On the other hand, even up to 10 h treatment of 96 h-old cells with cycloheximide or actinomycin D did not cause more than 20% or 32% loss of their enzyme activity respectively (Fig. 5*b*). Since the enzyme activity of untreated samples of 24 h-old cells was about 3 times that of 96 h-old cells, a rapid decline in the enzyme activity of 24 h-old cells appears to be due to inhbition of new synthesis of the enzyme protein and of its mRNA in these exponentially growing cells. On the other hand a limited response of 96 h-old cells to these metabolic inhibitors suggests that enzyme molecules were not actively synthesized by these confluent cells and that the enzyme activity in their plasma membranes represented the previously synthesized enzyme molecules.

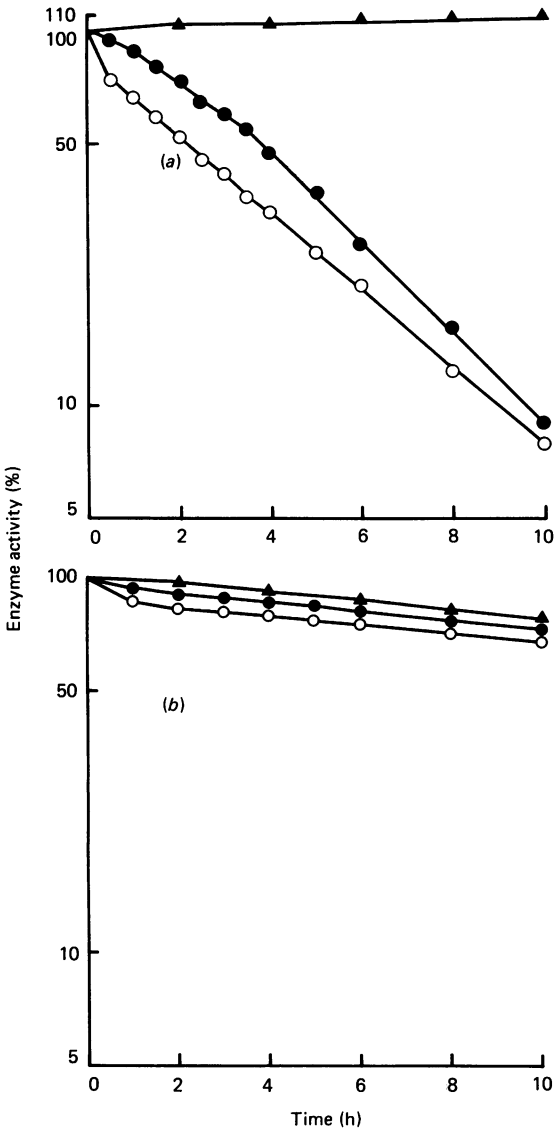

Fig. 5. Turnover rate of plasma-membrane-bound hyaluronate synthase in the cell

The 24 h- and 96 h-old cell cultures were treated with cycloheximide (10 μg/ml) or actinomycin D (5 μg/ml) for 0.5–10 h before preparation of plasma-membrane fractions. The plasma membranes thus prepared were assayed for their hyaluronate synthase activity. Plasma-membrane samples (50–100 μg) were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-Mg^{2+}, 5 mM-ATP, 100 mM-UDP-glucuronic acid, 100 μM-UDP-*N*-acetylglucosamine and 0.05 μCi of UDP-*N*-acetyl[^{3}H]glucosamine for 1 h at 37 °C. The enzyme activity was determined as described in the Materials and methods section. The values plotted are the averages for two experiments. (*a*) Plasma membranes of 24 h-old cells; (*b*) plasma membranes of 96 h-old cells. ▲, Control cells; ●, cells incubated with actinomcyin D; ○, cells incubated with cycloheximide.

Autophosphorylation of a high-M_r plasma-membrane-bound protein from [^{32}P]ATP during the hyaluronate-synthesis reaction

Incubation of freshly prepared plasma-membrane samples, from both 24 h- and 96 h-old cells, with

[γ^{32}P]ATP in 50 mM-Tris/HCl buffer, pH 7.1, resulted in the autophosphorylation of six proteins of apparent M_r 210000, 100000, 62000, 46000, 39000 and 27000 (Fig. 6*a*). However, when hyaluronate, precursors, i.e. UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine, were added to the reaction mixture, an additional high-M_r protein (M_r 450000) was also ^{32}P-labelled within 5 s of incubation reaction (Fig. 6*b*). The specific ^{32}P radioactivity of proteins of plasma membranes of 24 h-old cells was significantly higher than that of corresponding proteins of plasma-membrane samples of 96 h-old cells (Table 3). During this period no high-M_r macromolecules radiolabelled with ^{14}C derived from UDP-[^{14}C]glucuronic acid were detected (Fig. 6*b*). Incubation of plasma membranes for 1 h in the presence of [γ-^{32}P]ATP and UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine, however, resulted in the production of ^{14}C-labelled hyaluronate of $M_r < 2 \times 10^6$ along with the ^{32}P-labelling of 45000-M_r protein (Fig. 6*c*). Similar results were observed when UDP-*N*-acetyl[^{3}H]glucosamine instead of UDP-[^{14}C]glucuronic acid was used to monitor the synthesis of hyaluronate molecules (results not shown). The omission of UDP-glucuronic acid or of UDP-*N*-acetylglucosamine from the reaction mixture, which stopped hyaluronate synthesis, also abolished the [^{32}P]phosphorylation of 450000-M_r protein but did not affect the [^{32}P]phosphorylation of the other six membrane-bound proteins.

Preliminary observations on the role of 450000-M_r protein as a component of hyaluronate synthase

The native and ^{32}P-labelled 450000-M_r proteins isolated from plasma membranes of 24 h- and 96 h-old cells were examined for their ability to bind to hyaluronate precursors and to polymerize them into hyaluronate chains. The 450000-M_r protein, once auto[^{32}P]phosphorylated *in situ* in plasma membranes or in its isolated form (see Mian, 1986), remained ^{32}P-labelled. The incubation or washing of ^{32}P-labelled protein samples with buffer solution free of nucleotide sugar substrates or its precipitation with 5% (w/v) trichloroacetic acid did not remove covalently bound ^{32}P radioactivity from the protein. The nucleotide-sugar-substrate-binding activity of ^{32}P-labelled protein samples was greater than that of native protein, and the activities of protein samples prepared from 96 h-old cells were also higher than those of 24 h-old cells (Table 4). The addition of bivalent cations such as Mg^{2+}, Ca^{2+} and Mn^{2+} and of cyclic AMP and foetal bovine serum had no significant effect on the nucleotide-sugar-binding activity of the samples (Table 4).

The analysis of reaction products by gel filtration showed that isolated 450000-M_r protein samples did not synthesize high-M_r hyaluronate chains. However, the autophosphorylated protein samples of 24 h-old cells synthesized oligosaccharides of approx. M_r 5000 (Fig. 7) that could be completely hydrolysed to monosaccharides by the action of β-glucuronidase and β-*N*-acetylglucosaminidase. The corresponding protein from 96 h-old cells did not show any detectable oligosaccharide-synthesizing activity. The oligosaccharide-synthesis reaction was strictly dependent on Mg^{2+} (5 mM). The incubation of samples even up to 1 h, which increased the overall synthesis of 5000-M_r oligosaccharides, did not increase their M_r. A comparison of oligonucleotide–sugar-binding and hyaluronate-oligosaccharide-synthesizing activities showed that, among the seven ^{32}P-labelled proteins separated from plasma membranes of 24 h-old cells, only the 45000-M_r peak possessed these activities. Almost 96% of total hyaluronate-oligosaccharide-synthesizing activity of Nonidet P-40 extract of plasma

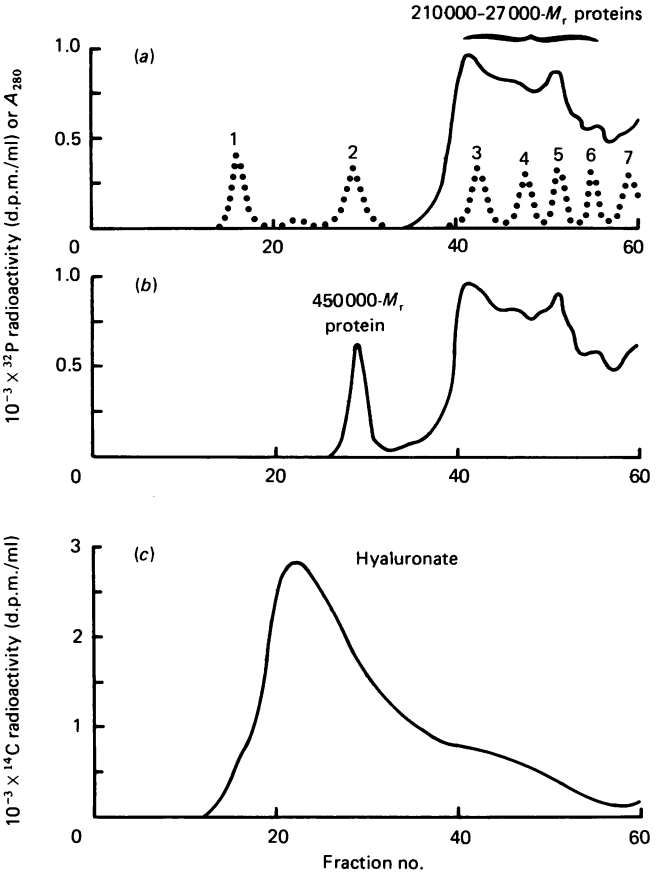

Fig. 6. [^{32}P]Phosphorylation of plasma-membrane proteins from [γ-^{32}P]ATP in reaction *in vitro*

The plasma membranes isolated from 24 h-old cells were incubated in 50 mM-Hepes buffer, pH 7.1, containing 5 mM-Mg^{2+}, 5 mM-ATP and 0.1 μCi of [γ-^{32}P]ATP (control) or in 50 mM-Hepes buffer, pH 7.1, containing 5 mM-Mg^{2+}, 5 mM-ATP, 0.1 μCi of [γ-^{32}P]ATP, 10 μM-UDP-glucuronic acid, 10 μM-UDP-GlcNAc and 0.05 M_rCi of UDP-[^{14}C]glucuronic acid (hyaluronate-synthesis reaction) for 5 s and 1 h periods at 37 °C. The reaction was terminated by the addition of SDS (1% w/v), and mixtures were subjected to gel filtration on precalibrated Sepharose CL-4B columns. (*a*) and (*b*) represent elution profiles of protein-bound ^{32}P radioactivity after 5 s incubation of plasma membranes under control and hyaluronate synthesis reaction conditions respectively. (*c*) represents ^{14}C radioactivity of hyaluronate synthesized during 1 h incubation of membranes under hyaluronate-synthesis reaction conditions described for (*b*). The ^{32}P radioactivity of column fractions in (*c*) closely resembled the ^{32}P-radioactivity elution profile shown in (*b*). Peaks 1–7 (·····, A_{280}) in (*a*) represent the elution of M_r markers during calibration of the column; 1, Blue Dextran ($M_r 2 \times 10^6$); 2, apoferritin monomer (M_r 450000); 3, rabbit muscle myosin (M_r 205000); 4, *E. coli* β-galactosidase (M_r 116000); 5, bovine serum albumin, (M_r 66000); 6, chicken egg albumin (M_r 45000); 7, carbonic anhydrase (M_r 29000).

Table 3. Specific ^{32}P radioactivity of some proteins solubilized from plasma membranes phosphorylated from [γ-^{32}P]ATP during control and hyaluronate-synthesis reaction

The plasma-membrane samples (approx. 500 μg) were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 2.5 mM-Mg^{2+}, 5 mM-ATP and 0.1 μCi of [γ-^{32}P]ATP in the presence of 10 μM-UDP-glucuronic acid and UDP-*N*-acetylglucosamine (hyaluronate-synthesis reaction conditions) or in the absence of these nucleotide sugar substrates (control reaction conditions). After incubation for 5 min at 37 °C, SDS (10%, w/v) was added to give a final concentration of 0.2%. The solubilized plasma-membrane samples were then subjected to gel filtration on Sepharose CL-4B columns calibrated with standard M_r protein markers as described in the Materials and methods section. The appropriate column fractions were collected, dialysed against 10 mM-Tris/HCl buffer, pH 7.1, and concentrated by freeze-drying before determination of the protein content and radioactivity. The results given are means ± S.E.M. for three separate experiments.

Approximate M_r of protein fraction	10^{-5} × Specific ^{32}P radioactivity of proteins fractionated from solubilized plasma membranes (d.p.m./mg of protein)				Ratio of specific ^{32}P radioactivity of protein samples of 24 h- and 96 h-old cells (hyaluronate-synthesis conditions)
	24 h-old cells		96 h-old cells		
	Control	Hyaluronate-synthesis conditions	Control	Hyaluronate-synthesis conditions	
27000	0.13±0.05	0.12±0.04	0.04±0.02	0.05±0.02	2.4:1
39000	0.34±0.07	0.35±0.06	0.13±0.03	0.11±0.04	3.2:1
46000	0.67±0.08	0.61±0.05	0.31±0.06	0.28±0.04	2.2:1
62000	1.42±0.16	1.47±0.21	0.82±0.13	0.73±0.11	2.0:1
100000	1.12±0.13	1.22±0.16	0.70±0.08	0.81±0.09	1.5:1
210000	1.84±0.25	1.72±0.28	0.68±0.09	0.65±0.05	3.5:1
450000	< 0.001	0.46±0.05	< 0.001	0.13±0.04	3.5:1

Table 4. Nucleotide-sugar-binding activities of high-M_r protein (M_r 450000) isolated from native and 'in-vitro'-phosphorylated plasma membranes of 24 h- and 96 h-old cells

The high-M_r protein (M_r 450000) was isolated from native and '*in-vitro*'-phosphorylated plasma-membrane fractions as described in Table 3 legend. The control samples (50–100 μg) were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 10 μM-UDP-glucuronic acid and UDP-*N*-acetylglucosamine in the presence of 0.01 μCi each of UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine. The test samples were prepared in a similar way by adding or omitting the agents listed below. The blanks were prepared at the same time with protein samples preheated at 100 °C for 2 min. All samples were incubated for 5 min at 37 °C with constant shaking before separation of the protein fraction by filtration. The protein samples were then subjected to radioactivity counting. The values given are means ± S.E.M. for five experiments.

Agent added	10^{-4} × Nucleotide-sugar-binding activity of 450000-M_r protein (d.p.m./mg of protein)			
	24 h-old cells		96 h-old cells	
	UDP-[^{14}C]GlcA	UDP-[^{3}H]GlcNAc	UDP-[^{14}C]GlcNAc	UDP-[^{3}H]GlcNAc
'*In-vitro*'-phosphorylated plasma membranes				
None (control)	3.26±0.42	3.82±0.36	5.01±0.46	5.83±0.62
+Mg^{2+} (5 mM)	3.18±0.36	3.75±0.29	4.78±0.51	5.72±0.67
+Ca^{2+} (5 mM)	3.21±0.36	3.80±0.32	4.87±0.62	5.65±0.72
+Mn^{2+} (5 mM)	3.17±0.58	3.70±0.69	4.81±0.80	5.52±1.08
+Cyclic AMP (100 μM)	3.27±0.49	3.76±0.62	4.78±0.73	5.72±0.95
+Foetal bovine serum (10%)	3.31±0.72	3.91±0.48	4.98±0.59	5.87±0.86
Native plasma membranes				
None (control)	1.51±0.12	1.72±0.19	3.21±0.42	3.91±0.32
+Mg^{2+} (5 mM)	1.45±0.08	1.81±0.11	3.16±0.48	3.78±0.45
+Ca^{2+} (5 mM)	1.53±0.36	1.84±0.32	3.26±0.51	3.76±0.39
+Mn^{2+} (5 mM)	1.46±0.82	1.38±0.72	3.08±0.95	3.26±0.65
+Cyclic AMP (100 μM)	1.52±0.28	1.92±0.36	3.24±0.92	3.75±0.85
+Foetal bovine serum (10%)	1.62±0.42	1.85±0.50	3.36±0.76	3.78±0.92

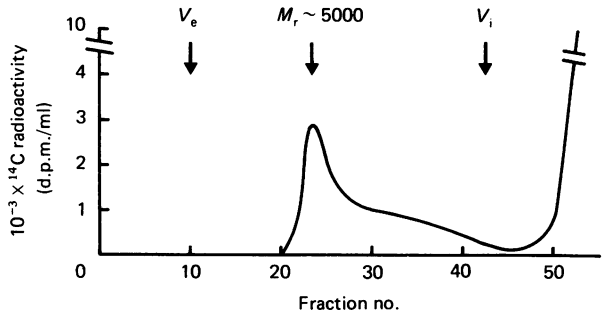

Fig. 7. Gel-filtration analysis of hyaluronate oligosaccharides synthesized by 450000-M_r protein isolated from plasma membranes of 24 h-old cells

The ^{32}P-labelled 450000-M_r protein was isolated from plasma membranes of 24 h-old cells. The protein sample (50 μg) was incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-ATP, 5 mM-Mg^{2+}, 10 μM-UDP-glucuronic acid, 10 μM-UDP-*N*-acetylglucosamine and 0.01 μCi of UDP-[^{14}C]glucuronic acid for 15 min at 37 °C. The sample was then heated at 100 °C for 2 min and subjected to gel filtration on Bio-Gel P-6 columns. The elution profile of ^{14}C radioactivity is plotted. V_e, excluded volume; V_i, included volume.

membranes of exponential-growth-phase cells was recovered in the 450000-M_r peak separated on the Sepharose CL-4B column. The specific hyaluronate-oligosaccharide-synthesizing activities of Nonidet P-40 extract and 450000-M_r peak were about $0.538 \pm 0.049 \times 10^4$ and $5.75 \pm 0.52 \times 10^4$ ($n = 7$) d.p.m. incorporated/15 min per mg of protein respectively.

DISCUSSION

The notion that hyaluronate synthase activity depends on the cell cycle (Tomida *et al.*, 1975) and cell density (Tomida *et al.*, 1975; Hronowski & Anastassiades, 1980; Prehm, 1980) has been derived from observations made on the rate of hyaluronate production either by intact cells or by their particulate fractions, including plasma membranes. The present study on the properties and reaction kinetics of plasma-membrane-bound hyaluronate synthase of cells during different growth phases was carried out with purified plasma-membrane fractions with a 24-fold-enriched hyaluronate synthase activity. The present data confirm that hyaluronate synthase activity residues exclusively in the plasma membrane of the cell (Appel *et al.*, 1979; Prehm, 1980, 1984; Mian, 1984). The hyaluronate synthase appears to be a plasma-membrane-bound enzyme and not a Golgi-membrane enzyme because all hyaluronate synthase activity was found in the plasma-membrane fraction, concentrated in a band of 1.145 g/cm^3 equilibrium density, which showed no detectable activity of *N*-acetylglucosamine galactosyltransferase (a Golgi-membrane marker enzyme). On the other hand, the *N*-acetylglucosamine galactosyltransferase was found in total cell homogenate and, in particular, in a fraction concentrated at 1.121 g/cm^3 equilibrium density on a linear sucrose density gradient where its specific activities were about 1.16 ± 0.24 and 85.5 ± 7.2 ($n = 7$) nmol of galactose transferred/h per mg of protein respectively. The fraction separated at 1.121 g/cm^3 equilibrium density, which presumably represent Golgi membranes, showed no detectable hyaluronate synthase activity. Although these obsrvations clearly demonstrate that the active form of hyaluronate synthase is a plasma-membrane-bound enzyme, they do not rule out the possibility that its pro-enzyme form may be of Golgi origin, since a number of plasma-membrane-bound enzymes have been reported to be Golgi-membrane-derived enzymes (Danielsen *et al.*, 1984).

The present work demonstrates that the specific activity of the enzyme, which was highest during the early exponential growth phase, decreased rapidly on entry of the cells into stationary growth phase. Whereas the enzyme preparations from both exponential- and stationary-growth-phase cells were similar in most of their general properties, they showed significant differences in their reaction kinetics and rate of elongation of hyaluronate chains. The differences in the reaction kinetic values, which must be considered with some reservations because of the particulate nature of the enzyme, suggest that there may exist some differences in the active site(s) of the enzyme from exponential- and stationary-growth-phase cells that affect the binding of nucleotide sugar substrates and the release of reaction product. The differences in the rate of elongation of hyaluronate chains by the enzyme from exponentially growing cells (50–60 disaccharide units/min) and from stationary-growth-phase cells (30–40 disaccharide units/min) may also point to the differences in the active site(s) of the enzyme molecule. The half-life of the enzyme in exponentially growing cells was fairly short: 2.2 h in the presence of cycloheximide and 3.8 h in the presence of actinomycin D (Davidson *et al.*, 1963; Davidson, 1963; Tomida *et al.*, 1974). The high total and specific activity of an enzyme molecule with an extremely short half-life depends largely on the continued synthesis of its mRNA and protein. The observed high activity of plasma-membrane-bound hyaluronate synthase in exponentially growing cells, which are actively engaged in both RNA and protein synthesis, compared with that in stationary-growth-phase cells is not at all surprising.

The observations that cyclic AMP, foetal bovine serum and Ca^{2+}, which stimulate hyaluronate synthesis in intact cells (Tomida *et al.*, 1975, 1977; Tajima *et al.*, 1981), had no stimulatory effect on hyaluronate synthase activity of isolated plasma membranes suggest that these agents must act via their effect on translational and post-translational cellular events that control synthesis and modification of the enzyme molecule rather than through their direct effect on stimulation of its activity.

Autophosphorylation of plasma-membrane proteins by endogenous protein kinase(s) is not an uncommon phenomenon (Joos & Anderer, 1979; Rubin & Rosen, 1975; Davies *et al.*, 1977; Branton, 1980). The present work also shows that some six proteins of M_r between 210000 to 27000 were routinely [^{32}P]phosphorylated on incubation of isolated plasma membranes with [γ-^{32}P]ATP. The higher specific ^{32}P radioactivity of plasma-membrane proteins of exponentially growing cells compared with that of stationary-growth-phase cells suggests that plasma membranes undergo covalent modification such as phosphorylation more actively during exponential growth phase rather than during stationary growth phase of the cells. The autophosphorylation of 450000-M_r protein from [γ-^{32}P]ATP under hyaluronate-synthesis reaction condition was, however,

of interest because of the possibility of participation of this protein in the enzyme reaction. For a protein molecule to function as an enzyme or as a constituent of an enzyme complex it is necessary that it must be able to bind to the substrate molecules and carry out catalytic reaction in a forward direction under favourable reaction conditions. The isolated ^{32}P-labelled 450000-M_r protein was found to possess efficient UDP-glucuronic acid- and UDP-*N*-acetylglucosamine-binding activities. Similarly the '*in-vitro*'-phosphorylated protein of plasma membranes of exponentially growing cells was also able to synthesize oligosaccharides of up to M_r 5000 and of about 12 glucuronosyl-*N*-acetylglucosamine disaccharide unit lengths.

The difference in nucleotide-sugar-substrate-binding and oligosaccharide-synthesizing activities of 450000-M_r protein from exponential- and stationary-growth-phase cells could be due to some modification of the protein molecule that increases its substrate-binding activity but abolishes its sugar-residue-polymerization activity in the stationary-growth-phase cells. The autophosphorylation of the protein molecule appeared to be an essential prerequisite for increasing its nucleotide-sugar-substrate-binding activity. Hence the elevated nucleotide-sugar-binding activities of protein isolated from stationary-growth-phase cells allude to the possibility that a higher number of protein molecules might be present in their phosphorylated form in the plasma membranes of these cells.

Besides hyaluronate-oligosaccharide-synthesizing activity of '*in-vitro*'-phosphorylated 450000-M_r protein of exponentially growing cells, both phosphorylated and native protein samples of exponential or stationary-growth-phase cells showed no high-M_r-(10^6–10^7)-hyaluronate-synthesizing activity. If this protein is a part of hyaluronate synthase complex, then the loss of its ability to synthesize high-M_r hyaluronate chains appears to be due to its removal from the rest of the ensemble. Perhaps, macromolecules neighbouring hyaluronate synthase molecule or this protein in the plasma membrane help to anchor the growing chain of hyaluronate while it is being elongated at the catalytic site of the enzyme.

Further characterization of this protein and the assessment of its role as a constituent of hyaluronate synthase complex are described in the following paper (Mian, 1986).

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