Regulation of heparan sulphate metabolism by adenosine 3':5'-cyclic monophosphate in hepatocytes in culture

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Freshly isolated rat hepatocytes maintained as monolayers in a serum-free medium synthesize sulphated glycosaminoglycans, most of which behave as heparan sulphate and are mainly distributed into intracellular compartments. Cyclic AMP, dibutyryl cyclic AMP, glucagon, noradrenaline, prostaglandin E₁, and theophylline, all drugs and hormones known to increase intracellular cyclic AMP concentrations, decreased the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate of intra-, extra- and peri-cellular pools. The inhibition mediated by dibutyryl cyclic AMP was dose-dependent and observed as early as 2 h after exposure to the drug. In the presence of 1 mM-dibutyryl cyclic AMP, incorporation of $^{35}\text{SO}_4^{2-}$ or [^{14}C]glucosamine into heparan sulphate was decreased to 40–50%, suggesting that dibutyryl cyclic AMP interfered with the synthesis of heparan sulphate. This was further supported by pulse-chase experiments, where dibutyryl cyclic AMP had no effect on the degradation of sulphated glycosaminoglycans. Heparan sulphates synthesized and secreted into the extracellular pool in the presence of dibutyryl cyclic AMP were smaller in size, whereas the degree of sulphation and molecular size of the heparan sulphate chains released by β -elimination from these proteoglycans were not different from control values. In the presence of 1 mM-cycloheximide, $^{35}\text{SO}_4^{2-}$ incorporation was decreased to 5%. Addition of *p*-nitrophenyl β -D-xyloside, an artificial acceptor of glycosaminoglycan chain synthesis, enhanced this incorporation to 18%. Dibutyryl cyclic AMP did not have any inhibitory effect on the synthesis of chains initiated on *p*-nitrophenyl β -D-xylosides. Incorporation of [^3H]serine into heparan sulphate was not affected by dibutyryl cyclic AMP, whereas the degree of substitution of serine residues with heparan sulphate chains was less in heparan sulphate synthesized in the presence of dibutyryl cyclic AMP, suggesting that cyclic AMP exerts its effect on the metabolism of sulphated glycosaminoglycans by affecting the transfer of xylose on to the protein core.

Biosynthesis of acid glycosaminoglycans by liver tissue or liver-derived cells has been demonstrated *in vivo* and *in vitro* (Yamamoto & Terayama, 1973; Akasaki *et al.*, 1975; Gressner *et al.*, 1977; Oldberg *et al.*, 1977). Oldberg *et al.* (1979) have recently isolated and characterized a heparan sulphate proteoglycan from a liver plasma-membrane preparation. Even though other acid glycosaminoglycans, such as chondroitin 4- and 6-sulphate, dermatan sulphate and hyaluronic acid, are reported to occur in liver (Delbrück, 1968; Gregoire *et al.*, 1972; Suzuki *et al.*, 1976; Dietrich *et al.*, 1976), primary cultures of hepatocytes synthesize predominantly

heparan sulphate (Oldberg *et al.*, 1977). Recent studies from this laboratory (Prinz *et al.*, 1980) showed that freshly isolated rat hepatocytes maintained in culture in a serum-free medium synthesize almost exclusively heparan sulphate at an almost constant rate for at least 3 days. Part of the heparan sulphate synthesized as proteoglycan is secreted into the extracellular compartment, whereas most of the material remains associated with the cell layer.

The liver is an important target organ for hormones and hormone mediated metabolic regulation. The physiological role of extracellular agents of hormonal and non-hormonal nature in the regulation of proteoglycan biosynthesis is well documented (Rendal *et al.*, 1972; Eisenbarth & Lebovitz, 1974; Kosher, 1976; Nero *et al.*, 1978).

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Schiller (1976) showed that the biosynthesis of proteoglycans in rat costal cartilage is influenced by hormones at the xylosyltransferase level. Kilgore *et al.* (1979) suggested that glucocorticoids and somatomedin may influence proteoglycan biosynthesis in embryonic chick cartilage by affecting the acceptor activity of the core protein.

The present study was undertaken to determine how the synthesis of heparan sulphate in primary cultures of hepatocytes is affected by hormones and cyclic nucleotides. Part of the present study has been reported elsewhere (Sudhakaran *et al.*, 1979).

Materials and methods

[1-¹⁴C]Glucosamine hydrochloride (sp. radioactivity 59 Ci/mol), L-[3-³H]serine (sp. radioactivity 105 Ci/mol) and ³⁵SO₄²⁻ (carrier-free, 5 Ci/mg of S) were obtained from Amersham, Braunschweig, Germany. Dibutyl cyclic AMP and cycloheximide were purchased from Boehringer-Mannheim, Mannheim, Germany. Glucagon, collagenase (EC 3.4.24.3) and chondroitinase ABC (EC 4.2.2.4) were from Sigma, München, Germany, and Pronase (trypsin; EC 3.4.21.4) was from Merck, Darmstadt, Germany. L-Adrenaline and ECTEOA-cellulose were purchased from Serva, Heidelberg, Germany, and L-noradrenaline was from Fluka, Neu-Ulm, Germany. Sephacryl S-300, Sepharose-4B CL and Percoll were obtained from Pharmacia, Uppsala, Sweden, Dowex AG1 (X2; Cl⁻ form; 200–400 mesh) was from Bio-Rad, München, Germany. Prostaglandin E₁ was kindly supplied by Upjohn, Kalamazoo, MI, U.S.A. Chondroitin sulphates of known mean molecular weight were a gift from Å. Wasteson, Biomedicum, Uppsala, Sweden. All other chemicals used were of analytical grade.

Cell culture

Female Wistar rats (200–250 g) were fed with water and Altromin (Lange, Lappe, Germany) *ad libitum*. Cells were isolated by the collagenase perfusion method of Seglen (1976) as described previously (Ullrich *et al.*, 1979). Hepatocytes were freed from non-parenchymal cells by sequential pelleting at 65 g for 1 min each in cold minimal essential medium (Eagle, 1959) containing 10% foetal calf serum (LS-Labor Service, München, Germany). The pellet obtained after three such centrifugations was subjected to isopycnic centrifugation in a Percoll gradient in sucrose (Ullrich *et al.*, 1978, 1979). Only preparations containing more than 90% viable cells as indicated by Trypan Blue exclusion test were used.

Hepatocytes (2.5 × 10⁶) in 5 ml of Eagle's Minimum Essential medium supplemented with 20% foetal calf serum, non-essential amino acids and

antibiotics (Cantz *et al.*, 1972) were seeded on 60 mm Falcon plastic dishes. They were incubated in CO₂/air (1:19) at 37°C for 5–6 h. The non-attached cells were sucked off and a chemically defined serum-free medium (Gorham & Waymouth, 1965), as modified by Fratanoni *et al.* (1969), was added. Hepatocytes cultured under these conditions maintained their characteristic morphology for at least 5 days. The hepatocytes were not contaminated with Kupffer cells as evidenced by the lack of phagocytosis of (a) ink (Günter Wagner, Hannover, Germany) and (b) fluorescein isothiocyanate-conjugated immunoglobulin G.

Determination of labelled glycosaminoglycans in hepatocytes

For the determination of ³⁵S- or ¹⁴C-labelled glycosaminoglycans, hepatocytes were incubated in 5 ml of serum-free medium containing either 5 μCi of ³⁵SO₄²⁻/ml or 5 μCi of [1-¹⁴C]glucosamine/ml and different compounds for 6 h unless otherwise indicated. The medium, the cell-surface-attached material releasable by 0.1% trypsin and the cell layer were obtained as described by Prinz *et al.* (1980). For isolation of [³⁵S]glycosaminoglycans portions of medium and trypsin solubilized material were precipitated in the presence of 0.5 mg of carrier chondroitin 4-sulphate/ml with cetylpyridinium chloride as described previously (Sudhakaran *et al.*, 1980). Unless otherwise mentioned the intracellular [³⁵S]glycosaminoglycans and cell protein were determined as described before (Fratantoni *et al.*, 1969; Lowry *et al.*, 1951).

¹⁴C-labelled glycosaminoglycans from all the three pools were subjected to digestion with Pronase (final concentration 0.75 mg/ml) in 0.1 M-Tris/HCl buffer, pH 7.8, at 37°C for 16 h and dialysed against water for 24 h at 4°C. Heparan sulphate was precipitated from the clear supernatant as above.

Heparan sulphate proteoglycans were labelled with [3-³H]serine by incubating the cultures for 16 h in the presence of 50 μCi of [3-³H]serine/ml in 5 ml of the serum-free medium modified by omission of unlabelled serine. ³H-labelled proteoglycans from the medium were precipitated with cetylpyridinium chloride as described above and fractionated over a ECTEOA-cellulose-23 column (0.5 cm × 5 cm) in 6 M-urea in sodium phosphate buffer (50 mM, pH 6.8) with a linear gradient of 0–1.5 M-NaCl prepared on an equal-weight basis of 50 g each. The heparan sulphates eluted were dialysed in the presence of 0.5 mg of chondroitin sulphate/ml prepared from bovine nasal cartilage (Hascal & Sajdera, 1969) against water for 24 h at 4°C. Dialysed sample was freeze-dried and subjected to Pronase digestion as above. Glycosaminoglycans from the clear Pronase digest were precipitated sequentially with cetylpyridinium chloride and ethanol.

Characterization of heparan sulphate

Heparan [^{35}S]sulphate was characterized by ion-exchange chromatography over a Dowex AG 1 (X2) column (0.5 cm $\times$ 5 cm), with a linear salt gradient made of 60 g of 0.1 M-NaCl and 60 g of 2.5 M-NaCl. Digestion with chondroitinase ABC and separation of the products by paper chromatography was by the method of Saito *et al.* (1968). Heparan sulphate chains were liberated from the proteoglycan by β -elimination at 37°C in 0.15 M-NaOH for 4 h. The molecular size of the proteoglycan and free heparan sulphate chains were determined by gel filtration over Sephacryl S-300 columns (1 cm $\times$ 97 cm) or Sepharose 4B CL columns (1 cm $\times$ 96 cm) equilibrated and eluted with 4 M-guanidine hydrochloride in 50 mM-acetate, pH 5.8.

Radioactivity was determined in a 2450 B liquid-scintillation counter (Packard, Frankfurt, Germany) with Instagel (Packard) as scintillation 'cocktail'.

Results

Effect of hormones and different drugs on heparan sulphate synthesis in hepatocytes

Synthesis of heparan sulphate by hepatocytes grown for 24 h in the presence and absence of different hormones such as glucagon, noradrenaline and prostaglandin E_1 and drugs such as dibutyryl cyclic AMP and theophylline, was assessed by separating and quantifying the ^{35}S -labelled polysaccharides by cetylpyridinium chloride and ethanol precipitation. The results of a typical experiment are shown in Table 1. In agreement with previous observations (Prinz *et al.*, 1980), freshly isolated

hepatocytes maintained in culture in a serum-free medium synthesized sulphated polysaccharides, most of which behaved as heparan sulphate as judged by susceptibility to HNO_2 treatment (Shively & Conrad, 1976) and resistance to chondroitinase ABC. About 23% of the ^{35}S -labelled polysaccharides were secreted and the remaining material was associated with the cell layer, of which 40% were integrated into the cell surface. The material associated with the cell surface was defined as that material that could be solubilized from intact cells with trypsin. Cyclic AMP, dibutyryl cyclic AMP, glucagon, noradrenaline, prostaglandin E_1 and theophylline decreased the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate of intra-, peri- and extra-cellular compartments. Total $^{35}\text{SO}_4^{2-}$ incorporation was decreased to about 40–80% of the controls at the concentrations of the different compounds tested. Dibutyryl cyclic AMP inhibited the incorporation of [^{14}C]glucosamine into heparan sulphate to a similar extent (results not shown).

Kinetics of dibutyryl cyclic AMP effect on $^{35}\text{SO}_4^{2-}$ incorporation

The inhibition mediated by dibutyryl cyclic AMP was dose-dependent. Concentrations as low as 1 μM decreased the $^{35}\text{SO}_4^{2-}$ incorporation into the heparan sulphate of the three pools to 72% of that of controls (Fig. 1). The degree of inhibition was more pronounced in the intracellular pool than in the other pools. The inhibition exerted by dibutyryl cyclic AMP was not due to its catabolic products as neither butyrate nor adenosine at appropriate concentrations had any inhibitory action (results not shown).

The degree of inhibition was constant over the

Table 1. Influence of various compounds on heparan [^{35}S]sulphate synthesis in primary cultures of rat hepatocytes. Cells were incubated for 24 h in the presence of different compounds and harvested for analysis of heparan sulphate. The values represent the means of duplicates from two dishes. Single values deviated from the mean by less than: $\pm 0.5\%$, *; $\pm 2\%$, †; $\pm 3\%$, ‡; $\pm 5\%$, §; Values given in parentheses are the percentages of control values.

Substance added	Heparan [^{35}S]sulphate (c.p.m./mg of protein)			
	Intracellular	Cell surface	Extracellular	Total
—	171 300 (100)	120 600 (100)	89 500 (100)	381 400†
Cyclic AMP (1 mM)	95 300 (55.6)	48 460 (40.1)	42 280 (47.2)	186 040‡
Dibutyryl cyclic AMP (1 mM)	95 240 (55.6)	69 100 (57.3)	31 410 (35.1)	195 750†
Prostaglandin E_1 (10 μM)	150 920 (88.1)	89 850 (74.5)	58 800 (65.7)	299 570†
Noradrenaline (2 μM)	168 050 (98.1)	91 780 (76.1)	56 740 (63.4)	316 570§
Theophylline (0.5 mM)	138 410 (80.8)	82 490 (68.4)	49 050 (54.8)	268 950*
Glucagon (0.1 μM)	114 090 (66.6)	74 890 (62.1)	45 740 (51.5)	234 720§

Fig. 1. Dose dependence of dibutyryl cyclic AMP on $^{35}\text{SO}_4^{2-}$ incorporation into heparan sulphate

Cells were incubated in the presence of various concentrations of dibutyryl cyclic AMP in $^{35}\text{SO}_4^{2-}$ -containing medium for 6 h and then harvested. Proteoglycans in the cellular, pericellular and extracellular pools were determined and the sum of the three pools was plotted. The values are means for duplicates with the range indicated by the vertical bars.

whole range of periods of incubation (up to 48 h) tested. The inhibitory effect was observed in the intracellular pools as early as 2 h, the minimum time required to study the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate in hepatocytes (Fig. 2). Incubation of hepatocytes with 1 mM-dibutyryl cyclic AMP for 2 h before labelling did not produce any additional effect. The inhibitory effect of dibutyryl cyclic AMP was reversible within 24 h after removal of the drug. Pulse-chase experiments, where cells prelabelled for 36 h with $^{35}\text{SO}_4^{2-}$ were incubated for up to 24 h in unlabelled medium supplemented with 1 mM-dibutyryl cyclic AMP, showed no difference in the rate of loss of label from heparan sulphate of the intracellular pool (Fig. 3). This suggests that dibutyryl cyclic AMP does not affect the rate of degradation, but rather the rate of synthesis of heparan sulphate.

Nature and size of heparan sulphate synthesized in the presence of dibutyryl cyclic AMP

Several possibilities might account for a decreased

Fig. 2. Time dependence of dibutyryl cyclic AMP effect on $^{35}\text{SO}_4^{2-}$ incorporation into heparan sulphate

Cells were incubated for different time intervals either in the presence of 1 mM-dibutyryl cyclic AMP (●) or in its absence (O) in $^{35}\text{SO}_4^{2-}$ containing medium and then harvested after trypsin treatment. Incorporation of radioactivity into intracellular proteoglycans was determined and the means for duplicate experiments were calculated. The range is given where it exceeds the size of the symbols.

synthesis of sulphated proteoglycans, including decreased synthesis of core protein, inhibition of glycosyltransferase activity or limited availability of sugar nucleotides. ^{35}S -labelled heparan sulphate proteoglycans prepared from the extracellular pool were smaller in size in dibutyryl cyclic AMP-treated cultures than in controls (Fig. 4). This could be due to the formation of smaller heparan sulphate chains and/or less chains on the protein core. Dibutyryl cyclic AMP had no effect on the molecular weight of heparan sulphate chains prepared from the intra- (Fig. 5) and extra-cellular pools. Heparan sulphate chains released from the proteoglycans of the extracellular pool of both control and dibutyryl cyclic AMP-treated cultures were eluted from Dowex 1 (X2) columns at a salt concentration of 1.25 M-NaCl (Fig. 6). Therefore the length and the degree of sulphation of the heparan sulphate chains synthesized in the presence of dibutyryl cyclic AMP are not different from that of controls.

Effect of *p*-nitrophenyl β -D-xyloside

It is known that several substituted β -xylosides can penetrate various types of cells and act as artificial acceptors for glycosaminoglycan synthesis when protein synthesis is blocked (Robinson *et al.*,

Fig. 3. Loss of intracellular $[^{35}\text{S}]$ glycosaminoglycans in the presence of dibutyryl cyclic AMP

Hepatocytes prelabelled for 36 h were washed extensively with Hanks' balanced salt solution and incubated for different periods either in the presence of 1 mM-dibutyryl cyclic AMP (●) or in its absence (○). Intracellular $[^{35}\text{S}]$ glycosaminoglycans were determined after 6–48 h. The means for duplicates and the ranges were plotted.

Fig. 4. Gel filtration over Sepharose 4B Cl of extracellular heparan sulphate

Proteoglycans isolated from the extracellular compartment were applied over Sepharose 4B Cl and eluted with 4 M-guanidinium chloride in 50 mM-acetate, pH 5.8. Symbols: ○, control; ●, 1 mM-dibutyryl cyclic AMP. V_0 and $V_{\text{H}_2\text{O}}$ mark the elution volume of Blue Dextran and $^3\text{H}_2\text{O}$.

Fig. 5. Gel filtration over Sephacryl S-300 of intracellular heparan sulphate chains

Heparan sulphate chains isolated from the intracellular compartments after Pronase digestion and alkali treatment were applied over Sephacryl S-300 and eluted with 4 M-guanidinium chloride in 50 mM-acetate, pH 5.8. Symbols: ○, control; ●, 1 mM-dibutyryl cyclic AMP. The column was calibrated with Dextran Blue (V_0), chondroitin sulphate standards of mol.wt. 19 000 (V_{19}) and 12 400 ($V_{12.4}$) and $^3\text{H}_2\text{O}$ ($V_{\text{H}_2\text{O}}$).

1975; Galligani *et al.*, 1975). In primary cultures of hepatocytes, $^{35}\text{SO}_4^{2-}$ incorporation was decreased to less than 5% by 1 mM-cycloheximide. No further inhibition was detected if dibutyryl cyclic AMP was added to this system. At a concentration of 1 μM -cycloheximide, where a residual proteoglycan synthesis of 25% was observed, dibutyryl cyclic AMP produced a similar relative inhibition as in control experiments (results not shown). Addition of 1 mM-*p*-nitrophenyl β -D-xyloside to hepatocytes pre-treated with 1 mM-cycloheximide led to an increase in the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate from 5 to 18% of that of controls. When dibutyryl cyclic AMP was added to the above system, no inhibitory effect on the $^{35}\text{SO}_4^{2-}$ incorporation was observed. To make sure that the increased $^{35}\text{SO}_4^{2-}$ incorporation affected by the addition of the xyloside was due to an increased synthesis of heparan sulphate chains initiated on the artificial acceptor and not of heparan sulphate proteoglycan, the secreted ^{35}S -labelled material was applied to a column of Sephacryl S-300. More than 95% of the material secreted by hepatocytes in the presence of cycloheximide and xyloside was eluted

Fig. 6. Chromatography over Dowex AG 1 (X2) of extracellular heparan sulphate chains

Heparan sulphate chains isolated from the extracellular pool was applied over Dowex AG 1 (X2) and eluted with a linear gradient of NaCl (●). Other symbols: O, control; ●, 1 mM-dibutyl cyclic AMP.

at a volume corresponding to the molecular size of free heparan sulphate chains, and very little in the void volume (Fig. 7). The molecular size of the material synthesized in the presence of cycloheximide alone did not differ from that isolated from controls (for comparison, see Prinz *et al.*, 1980). Dibutyl cyclic AMP has no effect on the synthesis of heparan sulphate chains initiated on an artificial acceptor. It seems therefore likely that dibutyl cyclic AMP affects the initiation of heparan sulphate synthesis on its core protein.

Labelling of heparan sulphate with [3-³H]serine

To determine the effect of dibutyl cyclic AMP on the substitution of serine residues present in the protein core, extracellular proteoglycans were prepared from hepatocytes grown in the presence of [3-³H]serine. The proteoglycans were isolated by precipitation with cetylpyridinium chloride and chromatographed on ECTEOLA-cellulose-23 in the presence of 6 M-urea. The material adsorbed to the ion-exchange column was eluted in two broad peaks

Fig. 7. Gel filtration over Sephacryl S-300 of extracellular heparan [³⁵S]sulphate of hepatocytes treated with dibutyl cyclic AMP in the presence of cycloheximide and *p*-nitrophenyl β -D-xyloside

Heparan [³⁵S]sulphate isolated from the extracellular pool was applied over Sephacryl S-300 and eluted with 4 M-guanidinium chloride in 50 mM-acetate, pH 5.8. Hepatocytes had been incubated with 1 mM-cycloheximide (O), 1 mM-cycloheximide and 1 mM-*p*-nitrophenyl β -D-xyloside (●) or 1 mM-cycloheximide and 1 mM-*p*-nitrophenyl β -D-xyloside and 1 mM-dibutyl cyclic AMP (●).

with a linear NaCl gradient. One of the peaks was eluted at a salt molarity of 0.6 M-NaCl as was heparan [³⁵S]sulphate (Fig. 8). Dibutyl cyclic AMP affected neither amount nor the elution behaviour of the ³H-labelled material co-eluting with heparan [³⁵S]sulphate. The ³H-labelled material corresponding to the heparan [³⁵S]sulphate was excluded by a Sephacryl S-300 column as was heparan [³⁵S]sulphate from secretions of hepatocytes. After Pronase digestion of the material obtained from controls, 42% of the ³H radioactivity was recovered in the glycosaminoglycan fraction after sequential precipitation with cetylpyridinium chloride and ethanol. When the material from the dibutyl cyclic AMP-treated cells was assayed in an identical manner, only 20% of the radioactivity was recovered in the glycosaminoglycan fraction. In either case more than 70% of the ³H radioactivity present in the glycosaminoglycan fraction could be released by alkaline treatment. Thus the major part of the ³H radioactivity present in the glycosaminoglycan fractions of controls and dibutyl cyclic AMP-treated hepatocytes appears to reside in *O*-glycosidically linked serine residues. The results indicate (i) that almost 50% of serine residues in heparan sulphate proteoglycan are substituted by glyco-

Fig. 8. Chromatography of extracellular heparan sulphate on ECTEOLA-cellulose-23

Extracellular proteoglycans from dibutyryl cyclic AMP-treated cells (a) and from controls (b) grown for 16h in the presence of [^3H]serine (●) were obtained after cetylpyridinium chloride and ethanol precipitation, applied on a ECTEOLA-cellulose-23 column in 6M-urea in 50mM-sodium phosphate, pH 6–8, and eluted with a linear gradient of 0–1.5M-NaCl (▲). For comparison the elution behaviour of heparan [^{35}S]sulphate proteoglycan of a control is shown (○). The fractions containing the heparan sulphate were pooled as indicated. The ^3H radioactivity recovered in these fractions was 15800 c.p.m./mg of cell protein for dibutyryl cyclic AMP-treated cells (a) and 14900 c.p.m./mg of cell protein for the control (b).

saminoglycan side chains and (ii) that the degree of substitution of serine residues is decreased by dibutyryl cyclic AMP to approx. 50% of that of controls.

Discussion

Cyclic AMP acts as a second messenger that is released within the cells of target tissues after stimulation by the first messenger (hormones). The tissues exhibit characteristic responses to the enhanced concentrations of cyclic AMP produced by the hormones (Robinson *et al.*, 1968). There are several reports on the effect of cyclic AMP or its derivatives on the metabolism of proteoglycans in organ or cell cultures. Drezner *et al.* (1976) observed that drugs that increase intracellular cyclic AMP

concentrations such as dibutyryl cyclic AMP and theophylline increased incorporation of $^{35}\text{SO}_4^{2-}$ into chick embryo pelvic cartilage proteoglycans. Addition of dibutyryl cyclic AMP to culture fibroblasts enhanced the secretion of ^{35}S -labelled glycosaminoglycans (Goggins *et al.*, 1972). Increase in endogenous cyclic AMP was found to be associated with an enhanced secretion of glycosaminoglycans in embryonic mouse fibroblasts (Peters *et al.*, 1974; Schönhöfer *et al.*, 1974). Koyama *et al.* (1976) observed that treatment of rat fibroblast cells, derived from a hepatoma tumour, with cyclic AMP resulted in an increase in the production of hyaluronic acid, whereas synthesis of sulphated glycosaminoglycans was unaffected. All these reports show an increase in the incorporation of $^{35}\text{SO}_4^{2-}$ into glycosaminoglycans by increase in cyclic AMP concentrations. The stimulatory effect of the sulphation factor present in the serum on the incorporation of $^{35}\text{SO}_4^{2-}$ into embryonic chick cartilage, however, is inhibited by cyclic AMP (Rendal *et al.*, 1972).

Most cell types synthesize a broad spectrum of glycosaminoglycans *in vitro*. The almost exclusive synthesis of only one glycosaminoglycan type greatly facilitates the study of the hormonal regulation of the heparan sulphate metabolism by hepatocytes. At variance with the above mentioned reports, our study shows that hormones and other drugs that increase intracellular cyclic AMP concentrations decrease the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate synthesized by primary cultures of hepatocytes that are maintained in a serum-free medium. The inhibitory action of dibutyryl cyclic AMP was dose-dependent and observed as early as 2h after exposure to the drug, suggesting that dibutyryl cyclic AMP interfered with the synthesis of heparan sulphate. This was further supported by pulse-chase experiments, where dibutyryl cyclic AMP had no effect on the rate of degradation of heparan sulphate.

Little is known about the synthesis and assembly of heparan sulphate. It may be assumed to proceed almost in the same manner as the synthesis of chondroitin sulphate, where the synthesis of a core protein is followed by the initiation of chains on specific serine residues by the transfer of xylose by UDP xylosyltransferase. Glycosaminoglycan side chains are then completed by the sequential addition of the appropriate monosaccharides, sulphation and modifying reactions (for review see Rodén, 1970; Lindahl *et al.*, 1977). In accordance with this concept is the observation that the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate in primary cultures of hepatocytes is considerably decreased by inhibition of protein synthesis. It is inferred from the results reported in the present paper that hormones inhibit via cyclic AMP the synthesis of heparan sulphate by

inhibiting the initiation of the glycosaminoglycan chains. The results leading to that conclusion may be summarized as follows. (1) Heparan sulphate secreted into the extracellular pool in the presence of dibutyl cyclic AMP is of smaller molecular size. (2) The molecular size and degree of sulphation of heparan sulphate chains released from the proteoglycan by β -elimination are not affected by dibutyl cyclic AMP. (3) β -Xylosides may serve as initiator of heparan sulphate chain synthesis in the absence of protein synthesis. Under such conditions where the need for core protein and xylosyltransferase is eliminated, dibutyl cyclic AMP has no inhibitory effect on the heparan sulphate chain synthesis. (4) Incorporation of [^3H]serine into heparan sulphate is not affected by dibutyl cyclic AMP. But the amount of serine residues substituted with heparan sulphate chains was decreased to 50% of the control. This decrease was comparable with the inhibition brought about by dibutyl cyclic AMP on the incorporation of $^{35}\text{SO}_4^{2-}$ into heparan sulphate secreted into the extracellular pool.

These results suggest that in hepatocytes cyclic AMP interferes with the initiation of heparan sulphate chains during proteoglycan synthesis. This may be due either to an interference with the acceptor activity of the core protein or to a decrease in UDP xylosyltransferase activity. The present results do not allow us to distinguish between these two alternatives. Attempts to assay UDP xylosyltransferase in hepatocytes under conditions that allow determination in chondrocytes (Schwartz, 1977) were not successful.

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