The Sulphation of Chondroitin Sulphate in Embryonic Chicken Cartilage

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1. Whole tissue preparations and subcellular fractions from embryonic chicken cartilage were used to measure the rate of incorporation of inorganic sulphate into chondroitin sulphate *in vitro*. 2. In cartilage from 14-day-old embryos, [^{35}S]sulphate is incorporated to an equal extent into chondroitin 4-sulphate and chondroitin 6-sulphate at a rate of 1.5 nmoles of sulphate/hr./mg. dry wt. of cartilage. 3. Microsomal and soluble enzyme preparations from embryonic cartilage catalyse the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate into both chondroitin 4-sulphate and chondroitin 6-sulphate. 4. The effects of pH, ionic strength, adenosine 3'-phosphate 5'-sulphatophosphate concentration and acceptor chondroitin sulphate concentration on the soluble sulphotransferase activity were examined. These factors all influence the activity of the sulphotransferase, and pH and incubation time also influence the percentage of chondroitin 4-sulphate formed.

Cell-free preparations of embryonic cartilage catalyse the incorporation of sulphate into chondroitin sulphate (D'Abramo & Lipmann, 1957). It seems reasonable to assume that this sulphotransferase activity is responsible for the sulphation of CS* *in vivo*, even though the nature of the sulphate acceptor and the stage in the biosynthesis of CS-protein at which sulphation occurs is not known.

With a soluble fraction from embryonic cartilage Adams (1960, 1963) and Meezan & Davidson (1967*a,b*) showed incorporation of ^{35}S from inorganic [^{35}S]sulphate and [^{35}S]PAPS into CS. Concurrent formation of CS chains is not essential for incorporation provided that polysaccharide acceptor is present in the reaction mixture. Protein-free C4S, C6S and dermatan sulphate all serve as suitable acceptors.

DeLuca & Silbert (1968), on the other hand, have used a microsomal preparation from the same tissue to study incorporation of ^{35}S from [^{35}S]PAPS into endogenous CS. This fraction also catalyses the formation of polysaccharide chains from UDP-sugars (Silbert, 1963; Perlman, Telser & Dorfman, 1964). The sulphotransferase activity appears to be bound to the particles and, once again, is not dependent on the concurrent formation of CS

chains. Although the microsomal activity is low, it is sufficient to alter the sulphate content and the properties of CS synthesized from UDP-sugars in the presence of PAPS (Silbert & DeLuca, 1968).

Embryonic chicken cartilage synthesizes almost equal quantities of C4S and C6S (Mathews, 1965; Robinson & Dorfman, 1969), and it seemed possible that the two sulphotransferase preparations reported in the literature might synthesize different products, particularly since Meezan & Davidson (1967*b*) have reported that the soluble preparation synthesized predominantly C4S.

Suzuki, Trenn & Strominger (1961) have obtained partial separation of sulphotransferase activities, specific for C4S, C6S and heparan sulphate, from a solubilized enzyme preparation derived from hen oviduct.

The following study was therefore undertaken to determine whether the soluble and microsomal preparations from embryonic chicken cartilage differ in the type of sulphation they catalyse.

EXPERIMENTAL

Materials

[^{35}S]PAPS. Rat liver was homogenized in 5 vol. of chilled 0.15M-KCl-1.0mM-EDTA (adjusted to pH 7.0 with NaOH) and centrifuged at 0–4° for 1 hr. at 105000*g* in a Spinco model L ultracentrifuge (rotor 40). A sample of the resulting supernatant solution, containing 58.5 mg. of protein, was incubated for 20 min. at 37° in 10 ml. of medium containing (final concentrations) 7.5mM-ATP, 0.075mM- Na_2SO_4 , 9.0mM- MgCl_2 , tris-HCl buffer, pH 8.0 (0.125M

* Abbreviations: CS, chondroitin sulphate; C4S, chondroitin 4-sulphate; C6S, chondroitin 6-sulphate; PAPS, adenosine 3'-phosphate 5'-sulphatophosphate; Di4S, 3-(β -D-glucosyl-4-enuronosyl)-2-acetamido-2-deoxy-D-galactose 4-O-sulphate; Di6S, 3-(β -D-glucosyl-4-enuronosyl)-2-acetamido-2-deoxy-D-galactose 6-O-sulphate.

with respect to tris), and 5.0 mc of carrier-free $\text{Na}_2^{35}\text{SO}_4$. The reaction was stopped by heating in a boiling-water bath for 2 min.; the mixture was chilled in ice and precipitated protein removed by centrifuging. The supernatant solution containing [^{35}S]PAPS was mixed with 70 μmoles of unlabelled PAPS, prepared by the method of Banerjee & Roy (1966), and applied to a column (20 cm. $\times$ 1.5 cm. diam.) of Dowex 1 (X4; formate form; 200–400 mesh). PAPS (1.09×10^9 c.p.m.) was eluted with a gradient of ammonium formate and formic acid (Robinson, 1964) as a symmetrical peak of radioactivity well separated from inorganic sulphate (3.95×10^9 c.p.m.). It was adsorbed on and eluted from charcoal as described by Banerjee & Roy (1966) to yield finally 57.5 μmoles of PAPS with specific radioactivity approx. 12 mc/m-mole and a transferable-sulphate/adenosine ratio 0.75 when assayed by the colorimetric method of Banerjee & Roy (1966). Purified phenol sulphotransferase for this assay was a generous gift from Dr A. B. Roy.

Carrier CS. This was prepared as the potassium salt after papain digestion of epiphyseal cartilage as described by Telser, Robinson & Dorfman (1965). Unless otherwise stated, carrier CS from 14-day embryos was used. Di4S and Di6S were prepared separately by digesting C4S and C6S respectively with purified chondroitin sulphate-lyase (Robinson & Dorfman, 1969).

Reagents. Carrier-free $\text{Na}_2^{35}\text{SO}_4$ was obtained from The Radiochemical Centre, Amersham, Bucks. ATP, tris and crystalline papain were from Sigma Chemical Co. (St Louis, Mo., U.S.A.); Pronase was from Calbiochem (Los Angeles, Calif., U.S.A.) and all other reagents were of analytical grade unless otherwise specified. Chondroitin sulphate-lyase (EC 4.2.99.6) was prepared from *Proteus vulgaris* N.C.T.C. 4636 and purified as described by Robinson & Dorfman (1969).

Methods

Incorporation of [^{35}S]sulphate in ovo. Freshly laid eggs of White Leghorn or Black Australorp chickens were incubated for periods of 14–20 days at 38° in an electric incubator. Carrier-free $\text{Na}_2^{35}\text{SO}_4$ was injected into batches of ten eggs after 13, 15, 17 and 19 days respectively. Each egg received 0.51 μC in 0.1 ml. of water through a small hole in the shell. After 24 hr. at 38° the embryos were removed, and the epiphyses of femurs and tibiae were dissected free of soft tissues and digested with papain as described by Telser *et al.* (1965). The CS from each batch was isolated and assayed for C4S content.

Incorporation of [^{35}S]sulphate in vitro. (a) Incubation of epiphyses. Epiphyses from 30–50 embryonic chickens (14 days old) were dissected free of soft tissues and suspended in sulphate-free Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing (final concentrations) 55 mM-glucose, 0.05 mM-glutamine and 0.002% Phenol Red and buffered at pH 7.4 by equilibration with $\text{O}_2 + \text{CO}_2$ (95:5) at 38°. They were shaken with $\text{Na}_2^{35}\text{SO}_4$ in 5.0 ml. of Krebs–Ringer buffer for 2 hr. at 38° in $\text{O}_2 + \text{CO}_2$ (95:5). After incubation, the flasks were heated for 2 min. in a boiling-water bath and the entire mixture was digested with papain, dialysed against water and the CS isolated as described below.

(b) Cell-free preparations. Epiphyses from 40–50 embryonic chickens (14 days old) were homogenized in an

all-glass Duall tissue grinder (Kontes Glass Co., Vineland, N.J., U.S.A.) with 25 ml. of ice-cold 0.10 M-KCl–2.0 mM-EDTA adjusted to pH 6.0 with KOH. The homogenate was centrifuged at 10000g for 10 min. at 0–4° in the Sorvall RC2-B centrifuge (rotor SS-34) and the residue discarded. The supernatant solution was then centrifuged at 105000g for 60 min. in a Spinco model L ultracentrifuge (rotor 40) and the final supernatant fraction used without further treatment. The pellet of microsomal material was resuspended in KCl solution and washed once by recentrifuging at 105000g for 60 min.

Cell-free preparations were incubated at 37° for 30–60 min. with 0.1 mM-[^{35}S]PAPS and imidazole or tris buffer in a final volume of 1.0 or 2.0 ml. Duplicate incubations were performed and a boiled-enzyme control was employed with each experiment. The reaction was stopped by heating the tubes in a boiling-water bath for 2 min., after which they were chilled in crushed ice, diluted to 4.0 ml. with water and incubated with Pronase (1.0 mg./ml.) for a further 24 hr. at 37° in tris–HCl buffer, pH 8.0 (0.1 M with respect to chloride).

Isolation of CS. CS was precipitated from the Pronase digest or from papain digests, after dialysis against water, by dropwise addition of cetylpyridinium chloride. The precipitates were carefully washed with 0.1% cetylpyridinium chloride in 0.05 M-NaCl to remove unbound radioactivity and the CS was finally isolated as the calcium salt as described by Telser, Robinson & Dorfman (1966).

Digestion with chondroitin sulphate-lyase and assay of C4S content. The calcium salt of CS was digested with purified chondroitin sulphate-lyase as described by Robinson & Dorfman (1969). Samples of the digests were examined by paper chromatography on Whatman 3MM paper in 2-methylpropionic acid–2.0 M-ammonia (5:3, v/v) together with internal standards of Di4S and Di6S. The areas corresponding to the standard disaccharides on the dry chromatograms were located by examination under u.v. light and were cut out and assayed for radioactivity in a liquid-scintillation spectrometer; this method gave reliable results with mixtures of ^{35}S -labelled disaccharides. The percentage C4S formed was calculated as radioactivity (c.p.m.) in Di4S as a percentage of the total radioactivity (c.p.m.) in Di4S and Di6S. In every sample between 60 and 90% of the total radioactivity migrated with the disaccharides.

Density-gradient fractionation of the soluble fraction. Soluble fraction obtained after centrifuging at 105000g was made 1.85 molal with respect to CsCl ($\rho = 1.30$) by slow addition of solid CsCl at 0–4° and centrifuged for 18 hr. at 105000g in a Spinco model L ultracentrifuge (rotor 50–Ti). The tubes were cut into two sections, and all the polysaccharide was recovered in the bottom one-third of the tube and the enzyme in the top two-thirds. The enzyme solution was applied to a column (20 cm. $\times$ 2.3 cm. diam.) of Sephadex G-25 equilibrated with 0.1 M-KCl–1.0 mM-EDTA at 0–4° and the protein was eluted, free from CS and CsCl, in the void volume of the column. The peak tubes were combined and used as outlined in the text.

Analytical methods. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. Aqueous solutions and paper sections were counted in a mixture composed of 0.5 ml. of aqueous sample, 5.0 ml. of ethanol and 10 ml. of toluene containing 6.0 g. of 2,5-diphenyloxazole/l. and 0.15 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. Some counting was performed in a mixture

composed of 0.5 ml. of aqueous sample and 15 ml. of scintillation mixture prepared as described by Bruno & Christian (1961). In both systems the counting efficiency for ^{35}S was approx. 60%.

Glucuronyl content was assayed with the carbazole method of Dische (1947), and protein by the biuret reaction (Layne, 1957), with crystalline bovine serum albumin as standard.

RESULTS

Whole epiphyses from embryonic chickens were considerably more active in incorporating inorganic sulphate into CS *in vitro* than the minced cartilage preparations used in previous studies (Telser *et al.* 1965). The incorporation (in nmoles/hr./mg. dry wt.) of inorganic sulphate by whole epiphyses is independent of the concentration of inorganic sulphate in the medium (Table 1), a fact indicating that the isotopic label is not diluted appreciably by endogenous sulphate. The calculated rate of sulphation of CS was 1.5 nmoles of sulphate incorporated/hr./mg. dry wt. of cartilage. The incorporation was linear with time during the first 2 hr. of incubation and the percentage of C4S formed is independent of the incubation time (Table 1).

To confirm that the proportion of C4S formed *in vitro* is the same as that *in ovo*, eggs were injected with $\text{Na}_2^{35}\text{SO}_4$ as described under 'Methods' in the Experimental section, and after 24 hr. further incubation labelled CS was isolated from the epiphyses and assayed for C4S content. The results in Table 2 show that the percentage of C4S

formed *in ovo* differs only slightly from the corresponding percentage obtained by incubating cartilage *in vitro*; both sets of results show the same increase in the proportion of C4S formed with increasing age and both show that epiphyseal cartilage from 14-day-old embryonic chickens synthesizes almost equal quantities of C6S and C4S. It is not known whether these two isomers of CS occur as separate and distinct polysaccharides or as a hybrid form (Robinson, 1968).

The sulphation catalysed by soluble and microsomal fractions from embryonic cartilage was examined. Initially the optimum conditions for the transfer of ^{35}S from PAPS to CS were determined by using the soluble fraction. Table 3 shows that the optimum pH for sulphation catalysed by the soluble fraction is 6.0–6.5 in the three buffers employed and that the pH optimum for the microsomal fraction is also approx. 6.5. The latter preparation was much less active than the soluble fraction under the assay conditions employed.

The effect of pH on the percentage of C4S formed is also shown in Table 3. Over the pH range 5.0–7.0 the percentage incorporation into C4S is relatively constant, but extremes of pH have a marked effect on the result. This phenomenon, which indicates a differential effect of pH on the C4S sulphotransferase and C6S sulphotransferase activities, might reflect a direct effect on the enzymes or, alternatively, an effect on the stability of [^{35}S]PAPS under the incubation conditions. The latter situation is feasible if two sulphotransferases occur, which differ in their affinity for PAPS.

Table 1. Incorporation of [^{35}S]sulphate into CS by whole epiphyseal cartilage from embryonic chickens *in vitro*

Whole epiphyses from 30 chicken embryos (14 days old) were incubated in Krebs–Ringer buffer as described under 'Methods' in the Experimental section. Each flask received 15 epiphyses (approx. 25 mg. dry wt. of cartilage), $10\mu\text{C}$ of carrier-free $\text{Na}_2^{35}\text{SO}_4$ and added inorganic sulphate as shown. Labelled CS was isolated and assayed for [^{35}S]C4S content as described under 'Methods' in the Experimental section.

Incubation time (min.)	Added sulphate (mM)	Incorporation		Percentage of C4S formed
		(c.p.m./ flask)	(nmoles of sulphate)	
0	1.0	240	—	—
30	1.0	52300	17.9	53
60	1.0	115000	39.4	56
90	1.0	166000	56.9	59
120	1.0	205000	70.5	62
150	1.0	227000	77.8	62
180	1.0	264000	90.5	59
120	2.0	84000	58.0	—
120	5.0	43600	74.8	—
120	10.0	22100	76.5	—

Table 2. Comparison of the sulphation of CS by embryonic chicken cartilage *in ovo* and *in vitro*

Whole epiphyses were incubated for 2 hr. in Krebs–Ringer buffer with carrier-free $\text{Na}_2^{35}\text{SO}_4$ as outlined under 'Methods' in the Experimental section. Eggs were also injected with $\text{Na}_2^{35}\text{SO}_4$ and incubated for 24 hr. as outlined under 'Methods' in the Experimental section. Labelled CS was isolated from the epiphyseal cartilage after papain digestion and was assayed for [^{35}S]C4S content as described under 'Methods' in the Experimental section. N.E., Not estimated.

Embryo age (days)	Percentage of C4S formed		
	(A)	(B)	(C)
	Minced cartilage <i>in vitro</i> *	Whole epiphyses <i>in vitro</i>	Whole eggs <i>in ovo</i>
13	52	59	N.E.
14	52	56–62	44
16	62	N.E.	50
18	72	N.E.	59
20	N.E.	N.E.	74

* Results obtained by Robinson & Dorfman (1969).

Table 3. *Effect of pH on the incorporation of sulphate into CS by cell-free preparations of embryonic chicken cartilage*

Preparation A (1.8mg. of protein/ml.) was a soluble enzyme fraction prepared from epiphyseal cartilage of 27 chicken embryos homogenized in 25 ml. of 0.15M-KCl-1.0mM-EDTA-10.0mM-MgCl₂-10.0mM-mercaptoethanol adjusted to pH 7.4 with KOH. Preparation B was the same preparation stored for 12 days at -20°. Preparations C and D were separate fractions from epiphyseal cartilage of 29 embryos homogenized in 25 ml. of the same buffer. Preparation C (2.92mg. of protein/ml.) was a soluble fraction and preparation D was a microsomal fraction (2.64mg. of protein/ml.) resuspended in 12.5 ml. of buffer. Each tube contained, in a final volume of 1.0 ml., 0.6 ml. of enzyme, 0.2 μ mole of [³⁵S]PAPS, 1.0 mg. of carrier CS and buffer as shown. After 40 min. at 38° the tubes were heated in a boiling-water bath for 2 min., treated with Pronase and the CS was isolated and assayed for [³⁵S]C4S content as described under 'Methods' in the Experimental section.

Enzyme preparation and buffer	pH	Sulphate incorporation (nmoles/mg. of protein/hr.)	Percentage of C4S formed
Preparation A:	9.18	0.31	30
tris-HCl (0.05M	8.64	0.45	32
with respect to tris)	8.20	0.72	44
	7.68	1.32	62
	7.18	2.49	70
	6.69	3.19	75
Preparation B:	6.93	1.98	76
imidazole-acetate	6.50	2.04	80
(0.05M with respect	5.96	1.98	79
to imidazole)	5.45	1.47	74
	4.95	0.39	29
Preparation C:	5.72	1.72	62
imidazole-HCl	6.16	2.10	67
(0.1M with respect	6.70	1.69	63
to chloride)	7.18	1.07	51
	7.61	0.48	33
Preparation D:	6.16	0.42	59
imidazole-HCl	6.70	0.43	57
(0.1M with respect	7.18	0.31	38
to chloride)	7.61	0.19	5

Both soluble and microsomal fractions catalyse the sulphation of both the 4- and the 6-position.

Fig. 1 shows that the effect of ionic strength on this reaction is quite marked with an optimum value near 0.075. Fig. 2 shows the effect of added Mg²⁺ and Mn²⁺. It is apparent that both metal ions stimulate the incorporation and, further, that inorganic sulphate does not effectively dilute the [³⁵S]PAPS. Table 4 shows that the degree of incorporation is proportional to the enzyme concentration over the range studied, that added cysteine has no significant effect on the incorporation and that addition of CS, derived from cartilage

Fig. 1. *Effect of ionic strength on the incorporation of sulphate into CS. Soluble enzyme was incubated with [³⁵S]PAPS in imidazole-HCl buffer, pH 6.0 (5.0mM with respect to chloride), as outlined under 'Methods' in the Experimental section. Ionic strength was varied by addition of KCl.*

Fig. 2. *Effects of added MnCl₂ and MgSO₄ on the incorporation of sulphate into CS. Soluble enzyme was incubated with [³⁵S]PAPS in imidazole-HCl buffer, pH 6.0 and 1.0.08, as outlined under 'Methods' in the Experimental section: ○, experimental points with added MnCl₂; ●, experimental points with added MgSO₄.*

of 14-day embryonic chickens, stimulates the incorporation but has no significant effect on the percentage of C4S formed.

The effect of PAPS concentration is shown as a double-reciprocal plot in Fig. 3. The K_m value, obtained by plotting lines of least squares, is 0.042mM at pH 8.0 and 0.039mM at pH 6.0. In all the other experiments, the initial PAPS concentration employed was either 0.1 or 0.2mM, more than twice the K_m value.

Table 4. *Effects of enzyme concentration, added thiol and added CS on the incorporation of sulphate catalysed by a cell-free preparation of embryonic chicken cartilage*

Soluble enzyme (3.6mg. of protein/ml.) was prepared from epiphyseal cartilage of 50 chicken embryos homogenized in 30ml. of 0.1M-KCl-2.5mM-EDTA adjusted to pH 7.4 with KOH. Each tube contained, in a final volume of 2.0ml., 0.2 μ mole of [³⁵S]PAPS, 2.0 μ moles of MnCl₂, imidazole-HCl buffer, pH 6.0 (50 μ moles of chloride), together with enzyme, cysteine and CS as shown. Sufficient carrier CS was added to each tube at the end of the reaction to give a total of 20mg./tube. After 30 min. at 38° the tubes were heated in a boiling-water bath for 2 min., digested with Pronase and the CS was isolated and assayed for radioactivity and C4S content as described under 'Methods' in the Experimental section.

Enzyme added (ml.)	Addition	Sulphate incorporation (nmoles)	Percentage of C4S formed
1.00	Boiled-enzyme control	0.06	—
0.25	—	0.82	—
0.50	—	1.72	—
0.75	—	2.45	—
1.00	—	3.06	41
1.00	Cysteine (0.01 M)	2.96	—
1.00	CS (2.0mg.)	4.28	35
1.00	CS (5.0mg.)	4.58	34
1.00	CS (10.0mg.)	4.75	36
1.00	CS (20.0mg.)	4.96	43

Table 5. *Effect of time on the incorporation of sulphate into CS catalysed by a cell-free preparation of embryonic chicken cartilage*

Soluble enzyme (4.4mg. of protein/ml.) was prepared from epiphyseal cartilage of 47 chicken embryos homogenized in 25ml. of 0.1M-KCl-1.0mM-EDTA buffered at pH 6.0 with imidazole-HCl buffer (5.0mM with respect to chloride). Each tube contained, in a final volume of 2.0ml., 1.4ml. of enzyme, 0.2 μ mole of [³⁵S]PAPS, 10.0 μ moles of MnCl₂, 8.0mg. of carrier CS from 16-day-old embryos and imidazole-HCl buffer, pH 6.0 (50 μ moles of chloride). The reaction was stopped by heating the tubes in a boiling-water bath and the CS was isolated and assayed for [³⁵S]C4S content as described under 'Methods' in the Experimental section.

Incubation time (min.)	Sulphate incorporation (nmoles/mg. of protein)	Percentage of C4S formed
0	0	—
15	0.64	62
30	0.98	51
45	1.28	44
60	1.51	40
90	1.61	39
120	1.64	37

Table 6. *Effect of acceptor CS on the incorporation of sulphate catalysed by a cell-free preparation of embryonic chicken cartilage*

Epiphyses from 42 chicken embryos were homogenized in 25ml. of 0.1M-KCl-1.0mM-EDTA buffered with 5.0mM-imidazole-HCl, pH 6.0. Soluble enzyme (1.8mg. of protein/ml.), free from CS, was prepared by centrifuging in a density gradient of CsCl as outlined under 'Methods' in the Experimental section. Each tube contained, in a final volume of 4.0ml., 3.0ml. of enzyme solution, 0.4 μ mole of [³⁵S]PAPS, 20 μ moles of MnCl₂, imidazole-HCl buffer, pH 6.0 (100 μ moles of chloride), and CS derived from 16- or 18-day-old embryos as shown. After 30 min. at 38°, the tubes were heated for 2 min. in a boiling-water bath and treated with Pronase, and the CS was isolated and assayed for [³⁵S]C4S content as described under 'Methods' in the Experimental section.

Addition	Sulphate incorporation (nmoles/hr./mg. of protein)	Percentage of C4S formed
—	0.09	—
16-day CS (0.5mg./ml.)	1.37	37
16-day CS (1.0mg./ml.)	1.59	33
18-day CS (0.5mg./ml.)	0.96	41
18-day CS (1.0mg./ml.)	1.09	40
18-day CS (2.0mg./ml.)	1.05	36

Fig. 3. Effect of [³⁵S]PAPS concentration on the incorporation of sulphate into CS: ○, at pH 6.0; ●, at pH 8.0.

The incorporation of sulphate catalysed by the cell-free system is linear with time over a 40 min. period as shown in Table 5. Paper electrophoresis of the incubation mixture after 40 min. demon-

strated that by that time almost all the PAPS was hydrolysed to inorganic sulphate. The results also clearly show that the extent of 4-sulphation changes throughout the incubation period; it is greatest during the early stages of the reaction and falls off as the reaction proceeds. This observation indicates

that the sulphation pattern obtained with cell-free preparations is not completely reliable, but it is consistent with the occurrence of two CS sulphotransferases, specific for 4-sulphation on the one hand and 6-sulphation on the other, which differ in their K_m value for PAPS.

In most of these experiments the sulphate acceptor was the endogenous CS-protein present in the preparation. To examine the effect of added acceptor in greater detail the endogenous CS-protein was removed from the soluble enzyme preparation by density-gradient centrifugation as outlined under 'Methods' in the Experimental section. Table 6 shows that the activity of the purified enzyme is dependent on the presence of acceptor and that concentrations of 1.0 mg./ml. are sufficient to give maximum incorporation. The addition of excess of CS has little effect on the percentage 4-sulphation catalysed by the purified enzyme, but the origin of the CS appears to affect the activity of sulphation.

DISCUSSION

The biosynthesis of CS-protein in vertebrate cartilage is of interest because of the complex structure and high molecular weight of the final molecule. This raises problems of mechanism and control not encountered in the biosynthesis of less complex proteins and polysaccharides.

Previous studies (Silbert, 1963; Perlman *et al.* 1964) have demonstrated that a microsomal fraction from embryonic cartilage catalyses the transfer of glucuronic acid and *N*-acetylgalactosamine from the corresponding UDP-sugars into a glycosaminoglycan very similar to chondroitin. It seems probable that the biosynthesis of the entire CS-protein is associated with this subcellular fraction and is limited by the rate of biosynthesis of the protein portion of the molecule (Telser *et al.* 1965, 1966).

DeLuca & Silbert (1968) have shown that the microsomal fraction also catalyses the transfer of sulphate from PAPS into endogenous CS and that when PAPS is present during the polymerization of the sugar residues a more polyanionic product is formed (Silbert & DeLuca, 1968). Elegant studies on the biosynthesis of heparin (Silbert, 1967) indicate that the sulphation of this polysaccharide occurs closely after or simultaneously with the polymerization of the sugar chains and that both steps are catalysed by a microsomal fraction from mast-cell tumour.

The occurrence, therefore, of relatively high CS sulphotransferase activity in the soluble enzyme fraction from embryonic cartilage is not entirely consistent with this notion. DeLuca & Silbert (1968) have suggested that the low activity in the microsomal fraction relative to that in the soluble

fraction may be due to the very rapid degradation of PAPS catalysed by the former. The results of the present paper do not exclude this possibility and real activity in the microsomal fraction may be considerably higher than that obtained. The activity in the soluble fraction, however, when assayed under optimum conditions, was approx. 1.0 nmole of sulphate/hr./mg. dry wt. of cartilage, a value that is of the same order as the measured incorporation of sulphate in whole cartilage.

Both microsomal and soluble preparations catalyse the transfer of sulphate to positions 4 and 6 of *N*-acetylgalactosamine in CS. They therefore do not differ in the type of sulphation that they catalyse.

The results show that the nature of the added CS acceptor influences the activity of sulphate incorporation but does not appear to alter the proportion of C4S formed. This property of the acceptor probably depends, at least in part, on the degree of sulphation of the polysaccharide chains.

Meezan & Davidson (1967b) have reported that the most active acceptor fractions of CS from embryonic chick cartilage are those most deficient in sulphate content. Variation in the molecular weight of the CS appeared to have relatively little effect. Their study, however, was performed with an unknown PAPS concentration and at a pH far from the optimum. These factors may be responsible for their observation that in the presence of endogenous acceptor only the main product of sulphation is C4S, whereas in the presence of added protein-free CS acceptor it is C6S, a result that is not supported by this study. The same factors may underlie the differences in acceptor efficiency reported for different polysaccharides by Meezan & Davidson (1967a) and Adams (1960).

Because such factors as ionic strength, time of incubation, PAPS concentration, the nature of the sulphate acceptor and particularly pH can all affect the activity and even the type of sulphation observed, studies on the cell-free incorporation of inorganic sulphate into CS must be considered to be of questionable significance.

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