BBA 4081

ACID MUCOPOLYSACCHARIDES OF FROG CARTILAGE IN THYROXINE-INDUCED METAMORPHOSIS*

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(Received January 8th, 1963)

SUMMARY

1. In early stages of larval development, cartilage of *Rana catesbiana* tadpoles contains a mixture of acid mucopolysaccharides which are incompletely sulfated. The polymer content is represented by about 40% chondroitin sulfate-A-type disaccharides, 20% chondroitin sulfate-C-type disaccharides, and 40% chondroitin-type disaccharides. During maturation of tadpoles, the degree of sulfation increases toward a content of equal amounts of chondroitin sulfates A and C. Further changes in composition occur during adult development after metamorphosis, since adult cartilage contains 70% chondroitin sulfate A and 30% chondroitin sulfate C. These changes in composition are not unique to the frog and may reflect an aspect of a common vertebrate pattern in development.

2. [^{14}C]Acetate and [^{35}S]sulfate are readily incorporated into chondroitin sulfates of cartilage slices *in vitro*. Cartilage slices from tadpoles induced to metamorphose with thyroxine show up to 10-fold increases in ^{14}C incorporation and up to 30-fold increases in [^{35}S]sulfate incorporation. The ^{35}S incorporation is mainly into chondroitin sulfate A.

3. Preparations were separated by electrophoresis and by ion-exchange procedures into fractions of differing degrees of sulfation. [^{14}C]Acetate was incorporated mainly into low-sulfated fractions of normal cartilage and into the high-sulfated fractions of metamorphosing cartilage, while the specific activity remained highest in the low-sulfated fractions from both kinds of cartilage. It is probable that the biosynthesis of chondroitin sulfates of varying degrees of sulfation involves sequential addition of sulfate to intracellular polymers by specific sulfotransferases.

4. A possible direct effect of thyroxine on sulfation in cartilage slices was not detected. Thyroxine may possibly exert *in vivo* a selective stimulating effect on sulfation mechanisms involved in synthesis of chondroitin sulfates during metamorphosis.

Abbreviations: CS-A, chondroitin sulfate A, the linear polymer of 3-*O*-β-D-glucuronido-*N*-acetyl-D-galactosamine-4-sulfate; CS-C, chondroitin sulfate C, the linear polymer of 3-*O*-β-D-glucuronido-*N*-acetyl-D-galactosamine-6-sulfate.

* This report is based largely upon a dissertation submitted by L. DEC. HINDS to the Department of Biochemistry, The University of Chicago, in partial fulfillment of the requirement for a Master's degree. A preliminary report was presented at a meeting of the Federation of American Societies for Experimental Biology in Atlantic City, N.J., April 14–18, 1962.

INTRODUCTION

Biochemical differentiation in visual and respiratory pigments and in enzyme mechanisms of excretion has been shown to precede or accompany anatomical metamorphosis of vertebrates, particularly amphibia[1,2]. The anatomical and biochemical changes that have been observed in metamorphosis of the frog tadpole, broadly interpreted as a preparation for terrestrial life, seem to recapitulate the evolutionary transition of vertebrates from water to land. We are led to enquire whether connective tissues possess a similar pattern of development.

CS-A- and CS-C-type polysaccharides, frequently in admixture, are systematic components of adult vertebrate cartilage. The results of an initial survey indicate further that CS-C is the predominant component in cyclostomes and fish, while CS-A is the predominant component in members of the more recently evolved, terrestrial classes of vertebrates[3]. Biochemical changes in acid mucopolysaccharides during ontogenesis of cartilage in an intermediate class, the amphibia, are thus of particular interest.

METHODS AND MATERIALS

Treatment of tadpoles

Tadpoles of the giant bullfrog, *Rana catesbiana*, were purchased periodically from General Biological Supply House (Chicago) and from Mr. L. H. BABBIT (Petersham, Mass.). Only tadpoles of a single lot, generally of the same size and age, were used in any experiment. The average ratio of hind leg length to over-all length was used as a measure of maturity or of development during metamorphosis. The tadpoles were kept at room temperatures (20–25°) in a large tank with aquatic plants and snails. Tadpoles which were to be treated with thyroxine were placed in a wide jar to accommodate 5 tadpoles per l of water and 67 μg DL-thyroxine (from Nutritional Biochemical Corp.) per l added. A stock thyroxine solution was prepared fresh each time by dissolving 0.1 mg per ml 0.005 M NaOH. The tadpoles were measured every 2 days and fresh water and thyroxine added. After 7–8 days, changes similar to those of normal metamorphosis were evident: tail regression, growth of hind legs, enlargement of eyes, widening of mouth, appearance of apertures for front legs and, occasionally, appearance of front legs. Tadpoles which reached this stage were then used in experiments. Mortality was usually low if thyroxine treatment was not extended beyond 8 days.

Sulfate incorporation *in vivo* was investigated with the above metamorphosing tadpoles by placing them for 48 h in a solution containing 500 μC of carrier-free $^{35}SO_4$ (from Oak Ridge National Laboratories) and 67 μg DL-thyroxine per l of $5 \cdot 10^{-4}$ M Na_2SO_4. The control consisted of normal tadpoles in a similar solution without thyroxine. Most of the experiments were carried out *in vitro* using cranial cartilage from both normal and from thyroxine-induced tadpoles. The tadpoles were dissected in the cold, the cranial cartilage scraped clean, and the pooled samples kept moist during the dissection in cold medium of the following composition: 50 ml Earle's balanced salt solution, 11 ml distilled water, 1 ml 4 % glucose, 0.1 ml 0.2 M L-glutamine, 0.22 ml 0.1 M Na_2SO_4, 0.04 ml 0.2 % phenol red. This medium has the approximate osmolarity of frog plasma[4] and attains a pH of 7.4 when equilibrated with a mixture of O_2 and CO_2 gases in volume ratio 95:5. The final sulfate concentration was $1 \cdot 10^{-3}$ M.

A medium for [^{14}C]acetate experiments was prepared by adding 1.5 ml 0.12 M sodium acetate to 13.5 ml of the above medium prior to equilibration with CO_2.

The cartilage was blotted free of excess solution, minced by scissors and aliquots of 1.0 g each were placed in 50 ml ground glass stoppered erlenmeyer flasks containing 4 ml of medium per g cartilage. The flasks were gassed for about 4 min with the O_2 and CO_2 gas mixture and tracer added in 1 ml of medium. Specific activities of tracer in the medium was about $1 \cdot 10^9$ counts/min/mmole acetate and $5 \cdot 10^9$ counts per min/mmole sulfate. The flasks were gently shaken mechanically for 3 h at 20–25°.

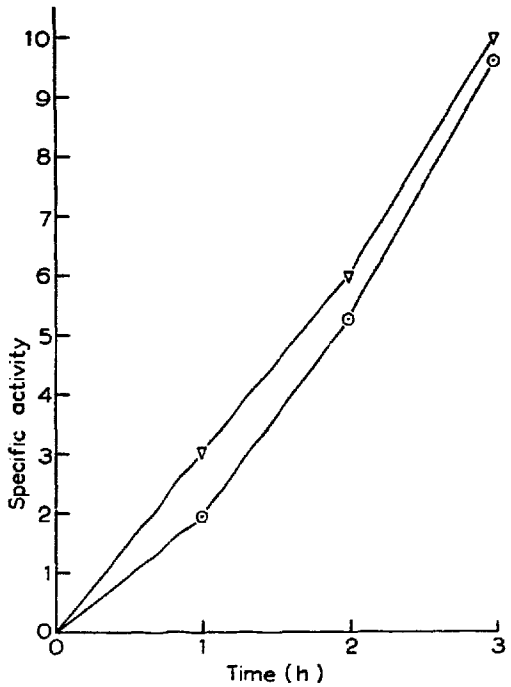


Fig. 1. Specific activity of incorporated ^{35}S into chondroitin sulfates of tadpole cartilage (O—O, counts/mg hexosamine × 10^{-3}) and of thyroxine-treated tadpole cartilage (▽—▽, counts/mg hexosamine × 10^{-4}).

The pH remained at 7.4 while the cartilage cells retained both their normal histological appearance and maintained their metabolic activity (Fig. 1) during this time. Controls contained 0.5% sodium iodoacetate (pH 7) added to flasks before gassing. A similar amount of sodium iodoacetate was introduced into the other flasks at the end of the incubation period. As previously reported by BOSTRÖM[5], iodoacetate was an effective inhibitor, reducing incorporation of tracer to 3% or less of the uninhibited incorporation. The incubation was immediately followed by enzyme digestion of the cartilage.

Isolation of acid mucopolysaccharides

Immediately after completion of incubation of cartilage minces, L-cysteine, equivalent to the iodoacetate present, was added to each flask. This was followed by 8 ml of 0.07 M phosphate buffer (pH 6.4), containing 0.01 mole cysteine and 7.4 g sodium EDTA per l, and 2 mg crystalline papain (EC 3.4.4.10) (Nutritional Biochemical Corp.) Digestion was carried out in an oven at 60° for 17 h. The residue, mainly bone salts, was removed by centrifugation and the supernatant dialyzed overnight against running tap water. The solution was made 0.2 N in NaOH by

addition of 20 N NaOH and kept at 4° for 24 h prior to neutralization with HCl. One-third vol. of cold 40 % trichloroacetic acid was added to the chilled solution which was kept at 4° for 1 h. The solution was clarified by centrifugation at 4° and dialyzed overnight against 100 vol. of 0.1 M K_2SO_4 (or 0.1 M sodium acetate when [^{14}C]acetate was used). Subsequent dialyses were against 100 vol. of 0.1 M NaCl followed by 0.01 M NaCl. The solution was concentrated 4-fold and the solution filtered. The acid mucopolysaccharides were then precipitated completely by a slight excess of 1 % cetylpyridinium chloride (K and K Laboratories, Inc.) in 0.04 N NaCl. The precipitate was separated by centrifugation and dissolved in 0.4 ml 2 N NaCl and 0.1 ml methanol. This solution was diluted with 1.6 ml of water and 6 ml ethanol added dropwise with shaking to precipitate the sodium salts of the acid mucopolysaccharides. After standing overnight at 4°, the precipitate was collected by centrifugation, washed with abs. alcohol and abs. ether and dried *in vacuo* with P_2O_5 overnight. The treatments with 0.2 M NaOH and with trichloroacetic acid were found necessary to reduce the molar ratio of nitrogen to hexosamine below 1.1, when maximum protein contamination was estimated at 2 %, and to give low tracer contamination. The yield of acid mucopolysaccharides was between 8 mg and 12 mg per g wet weight of cartilage from both normal and from metamorphosing tadpoles.

Fractionation by alcohol

Approx. 10 mg of acid mucopolysaccharide mixture was dissolved in 2.00 ml of 1.0 M calcium acetate buffer (pH 4.60) and fractionated with alcohol by a modification of a method used by MEYER *et al.*[6]. 1.00 ml abs. ethanol was added dropwise with stirring and the solution set in a bath at 2° with slow mechanical stirring overnight. The precipitate was collected by centrifugation at 2°, dissolved in water, dialyzed against 0.1 N NaCl and then against distilled water. A second fraction was obtained similarly by addition of 0.50 ml ethanol to the above supernatant. The resulting supernatant yielded a third fraction after further addition of 0.50 ml ethanol. The final supernatant contained up to 8 % of the total starting hexosamine. Analysis indicated that less than 50 % of this residual hexosamine might derive from keratosulfate, which would be expected to concentrate in the residue. The three precipitated fractions obtained differed primarily in the relative amounts of CS-A and CS-C. However, when the original mixture had a molar ratio of sulfur to hexosamine of less than 0.8, small differences in this ratio among the fractions could be detected. Solubility appeared to decrease with increase in degree of sulfation.

Electrophoresis

Electrophoresis was performed with an LKB apparatus using 2.5 × 36 cm cellulose acetate strips purchased from Colab Laboratories, Chicago Heights, Ill. The strips were soaked overnight in a pyridine–formic acid buffer (pH 3.0), containing 16.0 ml 90 % formic acid and 8.0 ml pyridine per l of solution. The strips were lightly blotted to remove excess buffer and 0.5 mg of acid mucopolysaccharides in 20 μl water applied in a 5 mm band 6 cm from one end of the strip. A voltage of 150 V was applied overall for 7 h. The strips were then dried in a horizontal position and stained by dipping for 2 min into a solution of 0.035 % toluidine blue in 65 % (v/v) ethanol–water. Backgrounds were de-stained by washing for about 5 min in two washes of 50 % (v/v) ethanol–water. After air-drying, the strips revealed a single, elongated,

elliptical spot (about 12 cm from the origin) which had a major diameter near 4 cm.

An 8 cm length of the strip was chosen and cut into 4 equal 2-cm wide sections. The front, middle, and back sections each contained, respectively, about 35 %, 50 %, and 15 % of the spot area. The remaining control section was only background stained. The sections were then placed in vials with 14 ml of solution containing 6.55 g 2,5-diphenyloxazole and 0.109 g 1,4-bis-2-5-phenyloxazolylbenzene (reagents purchased from Packard Instrument Company) per l of toluene and counted in a Packard Tri–Carb scintillation counter with a standard deviation of 5 % or less. The control section did not appreciably exceed the background count of the instrument obtained by omission of the section. Subsequently, the sections were washed with toluene, air-dried, and placed into 10 ml of 1 N HCl, which completely eluted the dye within 2 days. The absorbancy of the extract was measured at 630 mμ. Subtraction of the dye absorbancy of the control section yielded values of absorbancy proportional to the dye bound to acid mucopolysaccharide. The ratio of counts to absorbancy was taken as an approximate and relative measure of specific radioactivity.

This procedure has several limitations revealed by model experiments. When increasing amounts of a single radioactive preparation were present in spots on cellulose acetate with the spot area directly proportional to the amount of polysaccharide, the counts were also directly proportional to the amount of polysaccharide, whether stained or not. However, staining reduced the counts by nearly 75 %. In this instance, the amount of dye bound was directly proportional to the amount of polysaccharide. When the area was kept constant and the amount of polysaccharide increased, both counts and dye bound did not increase linearly with polysaccharide, although the ratio of counts to bound dye remained substantially constant. Thus, relative values of total radioactivity are less reliable quantitative measures than relative values of specific radioactivity. Based upon hexosamine, equivalent amounts of different preparations bound dye in proportion to their stoichiometric anionic charge per repeating period or, approximately, as $(1 + S/H)$, where S/H = molar ratio sulfur to hexosamine.

The electrophoretic mobility of a linear anionic polymer generally increases with increase in the electrostatic potential which is a function of the stoichiometric anionic charge per repeating period[7]. Thus, electrophoretic mobility should increase with increase in the value of $(1 + S/H)$ for a series of preparations. This was demonstrated with successive fractions obtained by use of ion-exchange columns.

Fractionation by resin

A glass column of 10 mm inside diameter was filled to a height of 200 mm with an analytical grade anion-exchange resin, AG-1 X2, 200–400 mesh, chloride form, obtained from California Corporation for Biochemical Research. 20–30 mg of a preparation of acid mucopolysaccharides in 3 ml of water was slowly adsorbed at the top of the column at a flow rate of about 1 drop/min. Elution of the column was carried out by various molarities of NaCl for periods of 24 h each at a rate allowing collection of 70–90 ml of effluent. Eluting solvents were as follows: A, water; B, 0.5 M; C, 1.1 M; D, 1.3 M, 1.4 M; F, 1.5 M; G, 1.6 M; H, 5.0 M. Effluents were dialyzed free of NaCl and evaporated at reduced pressure to a concentration near 1 mg polysaccharide per ml. Previous experience showed that this procedure did not effect a separation

of CS-A and CS-C in admixtures, but could separate preparations with low sulfate content intro fractions with increasing molar ratio of sulfur to hexosamine.

Assay of radioactivity

In addition to determination by scintillation assay, radioactivity was also measured with a gas-flow counter, Model C-110A of Nuclear Chicago, using a mixture of 98.7 % He and 1.3 % butane. Known amounts of polysaccharide, not exceeding 1 mg, were evaporated into 1.25-in diameter planchets. Planchets were subsequently counted with a standard deviation of 2 % or less.

Analytical procedures

Analytical values are based upon an anhydrous content of preparations determined by differential refractometry[8]. Otherwise, preparations were characterized by their content of hexosamine[9], with reference to a standard sample of highly purified CS-A. Glucuronic acid was determined by the method of DISCHE[10], and sulfur as sulfate after combustion with HNO_3 (ref. 11). Nitrogen was determined colorimetrically with Nessler's reagent following Kjeldahl digestion. Galactosamine and glucosamine were distinguished by chromatography[12].

The CS-C and CS-A contents of preparations, which could be completely digested[13] with hyaluronidase (EC 4.2.99.1), were estimated by a procedure[14] based upon the MORGAN–ELSON color given by tetrasaccharides in which the C-4 position of the amino sugar at the reducing end is unsubstituted. Such tetrasaccharides are derived from CS-C, but not from CS-A. When the molar ratio of sulfur to hexosamine, S/H, was less than unity, the fraction of CS-C in the mixture was given by $F - [1 - S/H]$ where F = fraction of tetrasaccharides yielding the MORGAN–ELSON color. The fraction of unsulfated tetrasaccharides was $1 - S/H$ and the fraction of CS-A was $1 - F$.

A deficiency in ester sulfate may occur in either CS-A or CS-C, or in both. It appears reasonable to assume that all hexosaminidic linkages are split by hyaluronidase with equal frequency and that the polymer chains are not too small so that end effects may be safely neglected. Then, simple statistical considerations lead to the above relations in a mixture for the relative amounts of the three different polymer repeating units of CS-C, CS-A and unsulfated CS-C (or CS-A). It was important to establish that prior to digestion, no appreciable amount of polymer without sulfate (detectable upon electrophoresis) appeared to be present. However, the possible presence of small amounts of hybrid polymer, containing both CS-C and CS-A repeating units within the same molecule cannot be rigorously excluded. Nevertheless, the separation of the nearly fully sulfated preparations into fractions of greatly differing CS-A contents indicates that CS-C and CS-A are largely present as discrete molecules. The values for CS-A content reported in this paper, thus, because of analytical limitations, refer strictly to the content of CS-A repeating units of a mixture. An analogous limitation applies to CS-C contents also.

RESULTS

Analysis of acid mucopolysaccharides

The compositions of typical acid mucopolysaccharide preparations are compared in Table I. The preparations contained galactosamine, with glucosamine not exceeding

TABLE I

ANALYSIS OF CHONDROITIN SULFATE PREPARATIONS

Sulfate and uronic acid are given as mole ratio to hexosamine. L/O, average ratio of leg length to over-all length; N, normal; T, thyroxine treated.

Source	*Maturity (L/O)*	*Sulfate*	*Uronic acid*	*CS-C (%)*	*CS-A (%)*
22S2 tadpole (N)	0	0.63	1.09	24	39
22M2 tadpole (N)	0	0.58	1.04	15	43
7N tadpole	0.08	—	1.02	—	32
7T tadpole	0.19	—	1.08	—	44
20N tadpole	0.11	0.93	1.07	47	46
20T tadpole	0.20	0.92	1.00	47	45
FS-1P frog	adult	1.02	0.96	30	70

3% of the total hexosamine. Hyaluronic acid or chondroitin were not detectable upon electrophoresis. Essentially complete digestion by hyaluronidase indicated that only CS-A- and CS-C-type polysaccharides were present in appreciable amount. During the period of normal tadpole maturation prior to metamorphosis, indicated approximately by the average ratio of leg length to over-all length, the content of CS-A remained nearly constant, while the degree of sulfation rose to near that representative of the adult frog. The CS-A content of acid mucopolysaccharides of adult cranial cartilage is, however, considerably higher than that for any tadpole cartilage. The apparent increase of CS-A content with thyroxin-induced metamorphosis was a borderline phenomenon at best.

Incorporation of [^{35}S]*sulfate*

A group of tadpoles were treated with thyroxine for 7 days to produce a change in the average ratio of leg length to over-all length from 0.11 to 0.20. Cartilage from this group and from an equivalent group of untreated tadpoles was used in an experiment designed to measure the incorporation of [^{35}S]sulfate. After extraction and purification from cartilage, the acid mucopolysaccharides were fractionated with alcohol. Aliquots of the original sample and of each fraction were assayed for CS-A and for radioactivity. These data are presented in Table II. Since the molar ratios of sulfur to hexosamine of the original preparations were near unity, the originals and their fractions apparently contained only CS-A and CS-C. The specific activities of CS-A and CS-C were then calculated by solving the appropriate simultaneous equations obtained from data on pairs of fractions with the greatest differences in composition

TABLE II

COMPOSITION AND SPECIFIC ACTIVITIES OF ALCOHOL FRACTIONS

N, normal; T, thyroxine treated; CS, chondroitin sulfate.

Sample	*Original*		*Fraction 1*		*Fraction 2*		*Fraction 3*	
	% CS-A	*Counts/min/mg CS*	*% CS-A*	*Counts/min/mg CS*	*% CS-A*	*Counts/min/mg CS*	*% CS-A*	*Counts/min/mg CS*
20N	46	1 700	69	3 580	24	1 520	32	1 240
20T	45	17 600	69	27 800	33	16 700	29	16 500

TABLE III

CALCULATED INCORPORATION OF [^{35}S]SULFATE INTO CS-A AND CS-C

N, normal; T, thyroxine treated.

Sample	*Fractions used*	*CS-A (counts/min/mg)*	*CS-C (counts/min/mg)*
20N	1.2	3 300	400
	1.3	5 600	800
20T	1.2	37 000	6500
	1.3	38 000	6500

as indicated in Table III. It is estimated that the errors in the specific activities of Table III are of the order of 500 counts/min/mg in sample 20N and 4000 counts per min/mg in sample 20T. In spite of these uncertainties, it appears that incorporation of [^{35}S]sulfate in normal cartilage is mainly into CS-A and that this preferential incorporation is greatly increased in cartilage from metamorphosing tadpoles.

Incorporation of [^{14}C]acetate and [^{35}S]sulfate

Parallel experiments were conducted with cartilage minces from both normal and metamorphosing tadpoles with additions of [^{14}C]acetate or [^{35}S]sulfate to the incubation flasks. The data in Table IV show that cartilage from metamorphosing tadpoles incorporates both tracers more rapidly than does cartilage from normal tadpoles. The increased incorporations over normal for ^{35}S showed a variation in the different experiments which may be related to the degree of metamorphic change as indicated by the increase in leg length:over-all length ratios. Incorporation of ^{35}S was increased relatively more than was incorporation of ^{14}C.

Electrophoretic fractionation

Samples from Expt. 30 were subjected to electrophoresis on cellulose acetate strips. Fig. 2 shows the distribution of tracer in fractions of differing mobility within each spot.

In cartilage of normal tadpoles, the bulk of the [^{14}C]acetate and the highest specific activity were associated with chondroitin sulfate of low mobility and low sulfate content (B), while the bulk of the [^{35}S]sulfate and the highest specific activity

TABLE IV

COMPARISON OF [^{14}C]ACETATE AND [^{35}S]SULFATE INCORPORATION INTO CHONDROITIN SULFATES

N, normal; T, thyroxine treated; L/O, average ratio of leg length to over-all length of tadpoles.

Expt.	*L/O*	*^{14}C (counts/min/mg)*	*^{35}S (counts/min/mg)*
21N	0.11	540	2 000
21T	0.31	1000	62 000
30N	0.08	250	870
30T	0.15	340	4 000
31N	0.07	400	1 060
31T	0.18	3500	18 200

TABLE V

ANALYSIS OF RESIN FRACTIONS OF EXPT. 31

N, normal; T, thyroxine treated; S/H, molar ratio sulfur to hexosamine. S/H values in parentheses are analytical values assumed to be approx. the same as that for a comparable fraction from a larger-scale fractionation of mucopolysaccharides from normal tadpole cartilage of Expt. 33. % yield values are based upon hexosamine.

	Fraction B			*Fraction C*			*Fraction D*			*Fraction EF*		
	% yield	*% CS-A*	*S/H*	*% yield*	*% CS-A*	*S/H*	*% yield*	*% CS-A*	*S/H*	*% yield*	*% CS-A*	*S/H*
$N^{35}S$	3.9	—	(0.25)	39	37	0.58	34	44	0.76	10.9	75	(1.00)
$N^{14}C$	3.6	—	(0.25)	34	37	0.62	37	63	0.71	9.8	76	(1.00)
$T^{35}S$	2.6	—	(0.25)	39	37	0.68	33	53	0.82	8.7	73	(1.00)
$T^{14}C$	3.5	—	(0.25)	39	34	0.57	34	65	0.84	8.6	74	(1.00)

TABLE VI

TOTAL COUNTS/MIN IN RESIN FRACTIONS OF EXPT. 31

N, normal; T, thyroxine treated.

	Fraction			
	B	*C*	*D*	*EF*
$N^{35}S$	150	10 100	7 100	8 900
$N^{14}C$	5 700	2 200	1 100	910
$T^{35}S$	1 000	47 000	105 000	113 000
$T^{14}C$	11 100	14 100	23 000	18 900

were found in the chondroitin sulfate of high mobility and high sulfate content (F). In cartilage of metamorphosing tadpoles, however, most of the [^{14}C]acetate appeared in the highly sulfated chondroitin sulfate (F), while the specific activity remained highest in the low-sulfated chondroitin sulfate (B). The distribution of [^{35}S]sulfate remained essentially unchanged.

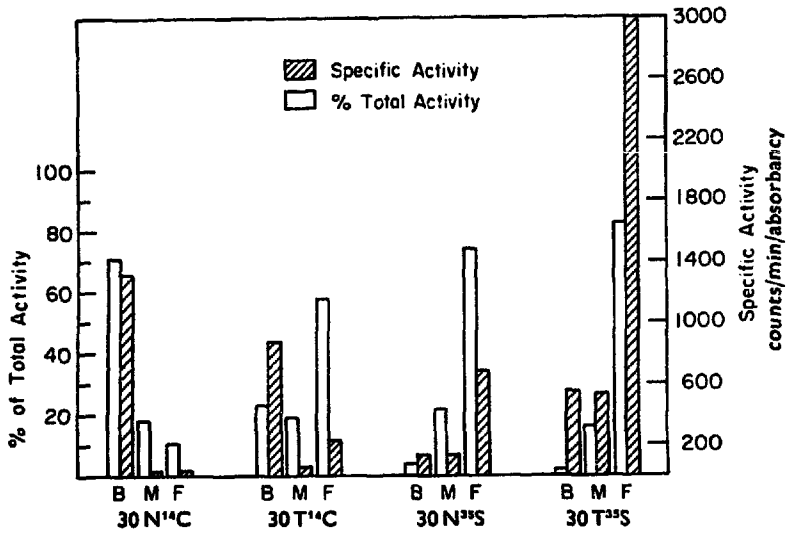


Fig. 2. Incorporation of tracer into electrophoretic fractions of samples from Expt. 30. The front (F), middle (M), and back (B) fractions are indicated.

Resin fractionation

In order to obtain amounts of acid mucopolysaccharide sufficient for chemical analysis, it was necessary to employ fractionation with Dowex-1 X2 resin. The starting materials were 20–30 mg each from Expt. 31. Electrophoresis of the samples had shown distributions of radioactivity very similar to those shown in Fig. 2. Fractions E and F were combined. The results are given in Tables V and VI.

Since Fractions B and EF were still inadequate for complete analysis, 100 mg of mucopolysaccharides in Expt. 33 were separated on four resin columns identical in dimensions to those used for Expt. 31. The yields of Fractions B, C, D and EF were,

Fig. 3. Electrophoresis of fractions of Expt. 33 obtained by resin fractionation of mucopolysaccharides of normal tadpole cartilage.

respectively, 7.4 mg, 32.8 mg, 40.5 mg and 11.1 mg; molar ratios of sulfur to hexosamine were, respectively, 0.25, 0.57, 0.76 and 1.00. Electrophoretic mobilities of these fractions increased with increase in molar ratio of sulfur to hexosamine as shown in Fig. 3. Similar mobilities were obtained for corresponding fractions of Expt. 31.

The distribution of radioactivity among the various resin fractions, differing in molar ratio of sulfur to hexosamine (Table VI), is essentially the same as that among the fractions previously obtained by electrophoretic separation.

DISCUSSION

Recent reviews[15-18] reveal our knowledge of the metabolism of sulfated mucopolysaccharides to be meager. The following diagram, Fig. 4, therefore represents solely a framework hypothesis for discussing the experimental data of this paper. Discussion is restricted to only three sulfated polymers, which may be approximated as n multiples of hexasaccharide units.

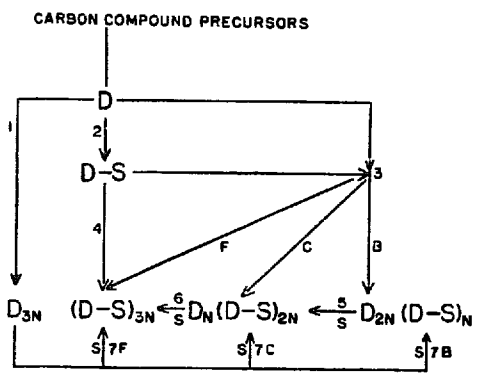


Fig. 4. Hypothetical scheme of biosynthesis. D, glucuronido-acetylgalactosamine disaccharide repeating unit; D-S, sulfated disaccharide repeating unit; B,C,F, polymer, corresponding approximately to fractions of Tables V and VI, as an n multiple of hexasaccharide units.

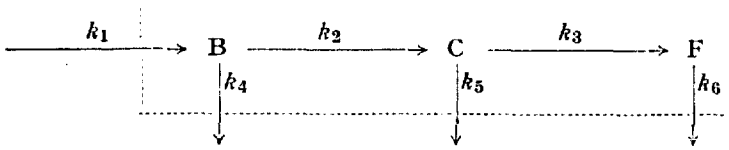
The scheme starts with a disaccharide (possibly bound as nucleotide) which is subsequently polymerized or sulfated and polymerized. Alternately, a plausible mechanism might start with monosaccharides which are subsequently polymerized, or sulfated and polymerized, in a regular alternating fashion. The existence of different sulfotransferase enzymes transferring sulfate from 3'-phosphoadenosine-5'-phosphosulfate to specific acid mucopolysaccharides as acceptors[19,20] suggests the probable importance of these enzymes in biosynthesis of sulfated acid mucopolysaccharides. Also, tracer sulfate may be transferred to acceptors varying from monosaccharides to high polymers, including desulfated high polymers, in cell-free solutions. However, no conclusions may be drawn from available data as to the relative importance of degree of polymerization in the biosynthesis of sulfated polymers at a cellular level. It is tacitly assumed here that the degree of polymerization of existing polysaccharides shown in the scheme is not further increased by mechanisms of addition of disaccharide units.

The present results suggest that sulfation of polymer molecules could be of major physiological significance. In the normal tadpole, ^{14}C is mainly incorporated into the low-sulfated Fraction B, possibly by Steps 1 and 7B or Steps 2 and 3B of

Fig. 4. However, very little ^{35}S appears in this fraction, the bulk of the sulfate appearing in Fractions C, D and EF via either Steps 2 and 4, Steps 5 and 6, or possibly by Steps 2, 3C and 3F or Steps 1, 7C and 7F.

If reversibility of the sulfation steps is excluded, as indicated by ADAMS AND RIENITS[21], then Fractions C and F possibly arise mainly via Steps 5 and 6 through sulfation of a pool of intracellular polymer of lower sulfate content, derived principally from polymer present prior to contact with tracers. This deduction acquires plausibility, since the exclusive operation of either Steps 2 and 3, or Steps 1 and 7, require that the incorporation of ^{35}S, after allowance for degree of sulfation, parallel the incorporation of ^{14}C into all fractions, a condition which is contrary to the experimental data. For example, in normal cartilage (Table VI) ^{14}C-activity of B is about 6 times that of EF, whereas ^{35}S-activity of B is very much less than 1.5 times that of EF. The above reasoning, however, depends upon the assumption of steady-state kinetics for the cellular compartment.

The open-system kinetics of the chondroitin sulfates of the intracellular compartment may be formulated from the following scheme:



In a steady state, it may be assumed that B is synthesized at a constant rate and that first-order kinetics apply to both the exit reactions of B, C and F from the cell compartment and to the sulfation of B and C. The following equations may be written:

$$\frac{dB}{dt} = k_1 - (k_2 + k_4)B = 0 \tag{1}$$

$$\frac{dC}{dt} = k_2B - (k_3 + k_5)C = 0 \tag{2}$$

$$\frac{dF}{dt} = k_3C - k_6F = 0 \tag{3}$$

$$B = \frac{k_1}{k_2 + k_4} \tag{4}$$

$$C = \frac{k_2}{k_2 + k_4} \cdot \frac{k_1}{k_3 + k_5} \tag{5}$$

$$F = \frac{k_3}{k_3 + k_5} \cdot \frac{k_2}{k_2 + k_4} \cdot \frac{k_1}{k_6} \tag{6}$$

$$\frac{C}{B} = \frac{k_2}{k_3 + k_5} \tag{7}$$

$$\frac{F}{C} = \frac{k_3}{k_6} \tag{8}$$

Upon thyroxine induction of metamorphosis, the incorporation of both tracer acetate and tracer sulfate is increased over normal. Although the bulk of the ^{14}C is now incorporated into the highly sulfated Fractions D and EF (Table VI), the specific

activity of ^{14}C remains highest in the low-sulfated Fraction B. From Eqns., 4, 5, and 6, it is seen that increase in k_1 alone does not lead to a change in the relative proportions of B, C, and F. However, an increase in sulfation rates, k_2 and k_3, results in increases in C, F and F/C and a decrease in B, provided that the exit constants remain unaltered. If the intracellular pool* approximates the total tissue pool in composition with C greater than B, it is an interesting consequence of Eqn. 7 that k_2 should exceed k_3.

Since the extracellular pool of sulfated polysaccharides arises from the intracellular pool of these compounds, it appears possible that the total tissue distribution of chondroitin sulfates with varying degrees of sulfation is related to the cellular rate of sulfation. Thus, an increase in rate of sulfation with metamorphosis (and possibly during prior maturation also) results in changes in the degree of sulfation of tissue chondroitin sulfates in the direction characteristic of adult frog cartilage. That specific sulfotransferases are involved is indicated by the preferential incorporation of ^{35}S into CS-A over CS-C (Table III). Additional data, particularly on the time sequence of incorporation of tracers, as well as on the influence of metamorphosis upon cellular permeability, pool sizes and enzyme levels, may help to clarify the biosynthetic mechanism.

The phylogenetic significance of the metamorphic changes in tadpole cartilage is not readily apparent. The increase in degree of sulfation and in the ratio of CS-A to CS-C with development is not a peculiarity of the frog, but has also been found in the chicken, rabbit, ox and possibly man[24]. Extension of studies to lower vertebrates may reveal that the increase in degree of sulfation in cartilage in development is a common pattern in the vertebrate phylum. However, adult cartilage of cyclostomes and of fish contains predominantly CS-C-type polysaccharides[3] while fish bone contains mainly CS-A-type polysaccharides[24].

An interpretation of the change in ratio of CS-A to CS-C with development must take into consideration the unusual biological status of adult cartilage. According to ROMER[25], it is highly probable that the presence of cartilage in adult vertebrates is not a primitive condition ancestral to bone, but indicates neoteny, the retention in the adult of an embryonic stage. Many living vertebrates, particularly amphibia, show less ossification than their ancestors of the same class. From this viewpoint, the increase in preferential synthesis of CS-A in cartilage of the metamorphosing tadpole does not appear to be a simple recapitulation of phylogenesis.

Since the sequence of change in composition during embryogeny is generally from low sulfation and low CS-A:CS-C ratio to high sulfation and high CS-A:CS-C ratio, retention in the adult of cartilage containing predominantly CS-A suggests retention of a late stage in vertebrate histogenesis. The well-known, close development of bone tissue in relation to cartilage matrices suggests that retention of "mature" cartilage may somehow reflect the comparatively great importance of internal bone and articular cartilage in the life of terrestrial vertebrates.

It is not known whether CS-A and CS-C may be synthesized by the same cell or are synthesized by different cell types. Nevertheless, it is apparent that hyaline cartilage may be topographically inhomogenous with respect to acid mucopolysaccharide composition[26], as well as to cellular type or cellular activity. LEBLOND

* The existence of pools of intracellular chondroitin sulfate has been shown by THORP[22] and by DZIEWIATKOWSKI[23]. For embryonic chick cartilage the intracellular pool possessed the same degree of sulfation and ratio of CS-A to CS-C as did the extracellular pool[22,17].

AND GREULICH[27] report that during transformation of hyaline cartilage of rat tibia into calcified cartilage, uptake into cells of both $^{14}CO_2$ and [^{35}S]sulfate is weak in hyaline cartilage, moderately increased in the zone of proliferation, and greatly increased in the zone of cell hypertrophy. It will therefore be important to determine whether biosynthesis of CS-A in tadpole cartilage may vary with type, location, and growth phase of cells. Some clarification of this picture may result when more is learned of the functional relationship of acid mucopolysaccharides to ossification.

The importance of thyroid hormone in bone development has been extensively documented[28,29]. FELL AND MELLANBY[30] found a direct effect of the hormone on maturation of cartilage of chick embryo limb buds in organ culture. We have investigated the possible direct effect of thyroxine on incorporation of [^{35}S]sulfate into cartilage slices of normal and metamorphosing tadpoles. In some experiments, tissues were treated with trypsin for 24 h at 4° to remove the metachromatic staining matrix, with and without the presence of thyroxine. In no case did thyroxine added in the range of 0.2–2.0 mg/l produce an effect. Such preliminary, negative experiments of relatively short duration, however, cannot be conclusive. The question of "direct" or "indirect" action of thyroxine upon metabolic activity of cells has not yet been resolved. Thyroxine is apparently capable of selectively stimulating the synthesis of specific proteins in the frog tadpole[2] possibly by rearrangement of specific RNA molecules[31]. The relatively large increases in tracer-sulfate incorporation compared to tracer-acetate incorporation may similarly be due to a selective effect of the hormone *in vivo* upon synthesis of sulfotransferases, or, alternatively, may be a "direct" effect upon cellular metabolism.

ACKNOWLEDGEMENTS

We are indebted to Dr. A. SAUNDERS for the histological examinations and to Mr. M. INOUYE and Mrs. L. ROTH for their analytical work. This investigation was supported by grants from the American Heart Association, Chicago Heart Association and the National Foundation.

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