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THE NATURE OF SULPHATION OF URONIC ACID-CONTAINING GLYCOSAMINOGLYCANS CATALYSED BY BRAIN SULPHOTRANSFERASE

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Abstract—A sulphotransferase system of rat brain catalyses the transfer of sulphate from 3′-phosphoadenosine 5′-phosphosulphate to the low-sulphated glycosaminoglycans isolated from normal adult human brain. These were shown to be precursors of higher-sulphated glycosaminoglycans by DEAE-Sephadex column chromatography and paper electrophoresis. Nitrous acid degradation and mild acid hydrolysis of enzymically-sulphated fractions further confirmed the presence of heparan sulphate in human brain.

A partially purified sulphotransferase preparation was obtained from neonatal human brain using chondroitin-4-sulphate as sulphate acceptor. This sulphotransferase catalyses the transfer of sulphate to the various uronic acid containing glycosaminoglycans. Heparan sulphate was the best sulphate acceptor followed by dermatan sulphate, *N*-desulphoheparin, chondroitin-4-sulphate and chondroitin-6-sulphate in decreasing order. Sulphotransferase obtained from 1-day-old rat, rabbit and guinea pig brain also had the same pattern of specificity towards various sulphate acceptors.

This sulphotransferase catalyses both *N*-sulphation and *O*-sulphation. Studies on the sulphotransferase obtained from both rat and human brain of various age groups indicate that the ratio of *N*-sulphation:*O*-sulphation decreases as the brain matures.

PREVIOUS work from this laboratory (BALASUBRAMANIAN and BACHHAWAT, 1964) showed the transfer of sulphate from 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to various glycosaminoglycans (GAG) such as heparan sulphate, dermatan sulphate, chondroitin-4-sulphate and chondroitin-6-sulphate by a cell free extract of young rat brain. The possibility that there are specific sulphotransferase enzymes for each sulphated GAG was indicated by SUZUKI, TRENN and STROMINGER (1961), BALASUBRAMANIAN, JOUN and MARX (1968) and DAVIDSON and RILEY (1960).

A number of GAG fractions containing uronic acid were isolated from normal human brain on DEAE-Sephadex columns and some of them had a low degree of sulphation (SINGH and BACHHAWAT, 1968). These low-sulphated fractions of GAG were found to be rich in heparan sulphate. Earlier work from Meyer's laboratory (MEYER, HOFFMAN, GRUMBACH and SAMPSON, 1959) also showed the presence of heparan sulphate in normal human brain. Recently, SINGH, CHANDRASEKARAN, CHERIAN and BACHHAWAT (1969) and CUNNINGHAM and GOLDBERG (1968) reported the presence of heparan sulphate in brains of various species. However, MARGOLIS (1967), SZABO and ROBOZ-EINSTEIN (1962) and CLAUSEN and HANSEN (1963) were unable to detect this GAG in nervous tissue. In the light of these contradictory reports, a study was undertaken using these low-sulphated GAG fractions from human brain

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Abbreviations used: PAPS, 3′-phosphoadenosine-5′-phosphosulphate; GAG, glycosaminoglycans; CPB, cetyl pyridinium bromide; *I*, ionic strength; DEAE, diethylaminoethyl; ISB, IISB & IISC; low sulphated GAG isolated from adult human brain having the constituents uronic acid, hexosamine and sulphate in the following ratios: ISB, 1:0·89:0·39; IISB, 1:0·95:0·72 and IISC, 1:0·93:0·92.

as acceptors of sulphate from PAPS in the presence of brain sulphotransferase to confirm the presence of heparan sulphate. It was also possible to show that these low-sulphated GAG fractions can act as precursors of the higher-sulphated GAG.

It was observed that in rat brain there is a change in sulphotransferase activity towards various acceptors with age (BALASUBRAMANIAN and BACHHAWAT, 1964). This study has now been extended to the human brain. It was shown that a partially purified system of sulphotransferase from human brain, free from endogenous acceptors, catalyses the sulphation of various glycosaminoglycans. It was further shown that this sulphotransferase preparation can catalyse both *N*- and *O*-sulphations. The rate of enzymic *N*- and *O*-sulphation was found to vary with age.

MATERIALS AND METHODS

ATP, bovine testicular hyaluronidase and protamine sulphate were obtained from Sigma Chemical Company, U.S.A. DEAE-Sephadex A-25, Sephadex G-25 and Sephadex G-75 were obtained from Pharmacia, Uppsala, Sweden. Chondroitin sulphate of bovine nasal septum was the product of Mann Research Laboratories, U.S.A. Chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate were obtained from Miles Laboratory Inc., Indiana. Heparan sulphate was kindly supplied by Professor Karl Meyer, Yeshiva University, New York. *N*-Desulphoheparin was a gift from Dr. A. S. Balasubramanian. Carrier free sodium [^{35}S]sulphate was obtained from Bhabha Atomic Research Centre, Bombay, India. All the other chemicals used were of analytical grade.

Human brain autopsy samples (without any neurological complications) of various age groups, viz. premature, neonatal, child (7- and 13-year-old), were made available through the courtesy of the Pathology Department. Tissues were cleaned free from meninges and adhering blood and used without freezing or storing. Guinea pig, rabbit and rat brain were obtained from animals bred in this laboratory.

Preparation of [^{35}S]PAPS. Active sulphate synthesizing enzyme was prepared from sheep liver according to the method of PANIKKAR and BACHHAWAT (1968). After ammonium sulphate fractionation, the enzyme was passed through Sephadex G-75 for desalting. [^{35}S]PAPS was prepared from ATP and inorganic [^{35}S]sulphate in the presence of active sulphate synthesizing enzyme as reported in an earlier communication (BALASUBRAMANIAN and BACHHAWAT, 1964). The identification of prepared [^{35}S]PAPS was confirmed by its ability to donate sulphate to *p*-nitrophenyl sulphate (BALASUBRAMANIAN and BACHHAWAT, 1961). These preparations contained about 50–60 per cent [^{35}S]PAPS and the rest was inorganic [^{35}S]sulphate which may have resulted from the degradation of [^{35}S]PAPS.

Carrier free [^{35}S]PAPS was used in most of the experiments described in this investigation. However, when net synthesis of sulphated GAG was studied, [^{35}S]PAPS was prepared using carrier sulphate with a specific activity of 300 μC/μmole of sodium sulphate.

Analytical methods. All radioactivity determinations for [^{35}S]sulphate incorporation studies were carried out in a Nuclear Chicago automatic counter. Protein concentrations were determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) using crystalline bovine serum albumin as standard. Uronic acid was determined by Dische's carbazole method (DISCHE, 1947) as modified by BITTER and MUIR (1962) using glucuronolactone as the standard. The hexosamines were assayed by the method of GATT and BERMAN (1966) after the various GAG fractions were hydrolysed with 6N-HCl at 100° in sealed tubes for 8 hr. The total sulphate content of GAG fractions was determined by the $BaCl_2$-gelatin method of DODGSON and PRICE (1962).

Assay of sulphotransferases. The assay mixture for enzymic sulphation of chondroitin-4-sulphate consisted of 20 μmoles of phosphate buffer pH 7·4, 1 μmole of $MgCl_2$, [^{35}S]PAPS [300,000–400,000 counts/min) 25 μg of chondroitin-4-sulphate (or other specific GAG acceptors) and enzyme protein in a total volume of 0·2 ml. After 2 hr incubation, it was deproteinized and processed as described in a previous paper (BALASUBRAMANIAN and BACHHAWAT, 1964). A Sephadex G-75 column (15 × 1·8 cm) equilibrated with 0·05 M-NaCl was used for separation of [^{35}S]GAG in the present investigation with better resolution than with Sephadex G-25.

Preparation of sulphotransferase. All enzyme preparations were carried out at 0–4° unless otherwise stated. A 20,000 *g* supernatant of brain homogenate was treated with 2% protamine sulphate to deplete the endogenous acceptors present in the brain, as described by BALASUBRAMANIAN and BACHHAWAT (1964). The supernatant fluid obtained from rat brain after protamine sulphate treatment was used as a source of sulphotransferase for experiments with low-sulphated GAG as well as for studies on specificity of sulphotransferase obtained from various animals. In case of human brain sulphotransferase of various ages, protamine sulphate supernatant was brought to 45 per cent

ammonium sulphate saturation by the gradual addition of solid ammonium sulphate with stirring. After stirring for 20 min, it was centrifuged at 10,000 *g* for 20 min. Both the precipitates and supernatant were collected separately. Ammonium sulphate saturation of the supernatant was raised to 65 per cent by further addition of solid ammonium sulphate in the same manner. Both 0–45 per cent and 45–65 per cent ammonium sulphate precipitates were dissolved in minimal amounts of 0·02 M-tris–HCl buffer, pH 7·4 (20–30 mg protein/ml), and they were dialysed for 8 hr against 50 vols. of the same buffer. The 45–65 per cent ammonium sulphate fraction was used for comparison of specific activity of the sulphotransferases obtained from brain of different age groups towards various GAG acceptors as well as for the study on the site of sulphate incorporation in the case of heparan sulphate.

Isolation of low sulphated GAG. Three low-sulphated GAG fractions containing uronic acid, designated as ISB, IISB and IISC, were obtained from normal adult human brain as described by SINGH and BACHHAWAT (1968). The uronic acid:hexosamine:sulphate ratio of ISB, IISB and IISC were 1:0·89:0·39, 1:0·95:0·72 and 1:0·93:0·92 respectively. However, to isolate the GAG complex bound to peptide, the processing of the brain was carried out similarly except for the omission of alkali treatment, and a similar GAG pattern was obtained. The non-alkali treated fraction contained a considerably greater amount of ninhydrin positive material as determined by Rosen's method (ROSEN, 1957) after 6 N-HCl hydrolysis at 140° for 3 hr. The fractions obtained after alkali treatment are designated as peptide-free ISB and peptide-free IISB. The low-sulphated GAG fractions were separately digested with testicular hyaluronidase according to the method of MATHEW and INOYE (1961) and undigested fractions designated as hyaluronidase-resistant ISB and hyaluronidase-resistant IISB were obtained by gel filtration on Sephadex G-25 as described by SINGH and BACHHAWAT (1965). All these fractions were used for the sulphate incorporation studies.

Enzymic sulphation of low sulphated GAG. All the sulphated GAG fractions were incubated at 37° with carrier [^{35}S]PAPS using protamine sulphate supernatant obtained from rat brain as the enzyme source.

For the characterization of enzymically sulphated [^{35}S]GAG, 8·4 nmoles (532,000 counts/min) of [^{35}S]PAPS, 68·0 nmoles uronic acid of acceptor GAG and an enzyme preparation from 5-day-old rat brain were incubated for 3 hr, to obtain [^{35}S]GAG with higher radioactivity. The [^{35}S]GAG obtained after gel filtration was dialysed against distilled water for 8 hr and then used for further identification as follows. A portion of isolated [^{35}S]GAG was precipitated with CPB in the presence of celite, extracted with increasing concentrations of NaCl, and precipitated with alcohol (80%) as reported previously (SINGH and BACHHAWAT, 1968). The GAG fractions were further subjected to DEAE-Sephadex column chromatography and eluted with various concentrations of NaCl. The radioactivity of each fraction was measured. ISB and IISB were designated as [^{35}S]ISB and [^{35}S]IISB after enzymic sulphation *in vitro*.

Nitrous acid treatment. [^{35}S]GAG obtained after enzymic sulphation was subjected to nitrous acid degradation at room temperature for 80 min, using the method of LAGUNOFF and WARREN (1962) for the determination of heparan sulphate in which the free amino group and *N*-sulphate of hexosamines are susceptible to the action of nitrous acid, giving rise to anhydromannose with the concomittant rupture of adjacent glycoside bonds. The reaction products resulting from nitrous acid degradation were fractionated by gel filtration on Sephadex G-25 (20 × 1·8 cm) equilibrated with 0·02 M-NaCl.

Determination of N- and O-sulphation. [^{35}S]GAG fractions obtained after enzymic sulphation were hydrolysed in sealed ampules at 100° in 0·04 N-HCl for 90 min for desulphation of the sulphamino groups (LAGUNOFF and WARREN, 1962; DANISHEFSKY, 1965). After hydrolysis, the incubate was neutralised with NaOH and a portion was passed through a Sephadex G-25 column (20 × 1·8 cm) equilibrated with 0·02 M-NaCl to separate the [^{35}S]GAG and inorganic [^{35}S]sulphate. When chondroitin [^{35}S]sulphate mixture was subjected to hydrolysis under the same conditions, 80 per cent of the radioactivity remained with the GAG.

Paper electrophoresis. Paper electrophoresis of the isolated fractions obtained after enzymic sulphation was carried out using 0·1 *I* lithium sulphate (FOSTER and PEARCE, 1961), and was followed by radioautography. The paper was stained with 0·5% alcian blue in 3% acetic acid.

RESULTS

Enzymic sulphation of low-sulphated-GAG. As shown in Table 1, there was a net transfer of sulphate from PAPS to the two low-sulphated GAG acceptors. Fraction ISB which had a sulphate/uronic acid ratio of 0·39 was the best acceptor. Sulphate incorporation into IISB was comparatively low and it had a higher sulphate content (0·72 μmole sulphate/μmole of uronic acid). In the case of fraction IISC, sulphate incorporation was almost negligible and the ratio of sulphate/uronic acid was 0·92.

TABLE 1.—ENZYMIC SULPHATION OF LOW-SULPHATED GAG

Acceptor GAG	[^{35}S]Sulphate incorporated	
	Counts/min	nmoles
Experiment I		
ISB	6900	0·03
IISB	2500	0·01
IISC	640	0·003
Chondroitin sulphate from bovine nasal septum	1400	0·006
Experiment II		
ISB	18,200	0·29
Peptide-free ISB	15,800	0·25
IISB	8800	0·14
Peptide-free IISB	10,900	0·18
Hyaluronidase resistant		
ISB	17,600	0·28
Peptide-free ISB	32,100	0·52
IISB	26,700	0·14
Peptide-free IISB	21,500	0·18

Incubation mixture for experiment I consisted of 40 μmoles of tris–HCl, pH 8·0, 2 μmoles of $MgCl_2$, 0·6 nmole (131,500 counts/min) of [^{35}S]PAPS, 50 μg of sulphate-acceptor GAG and 0·56 mg of enzyme protein obtained from protamine sulphate supernatant of 5-day-old rat brain in a total volume of 0·3 ml. For experiment II, it consisted of 40 μmoles of tris–HCl, pH 8·0, 2 μmoles of $MgCl_2$, 12 nmoles (760,000 counts/min) of [^{35}S]PAPS, 14·0 nmoles uronic acid of sulphate-acceptor GAG and 1·2 mg of enzyme protein obtained from protamine sulphate supernatant of 15-day-old rat brain in a total volume of 0·4 ml. The incubation was carried out for 2 hr at 37°.

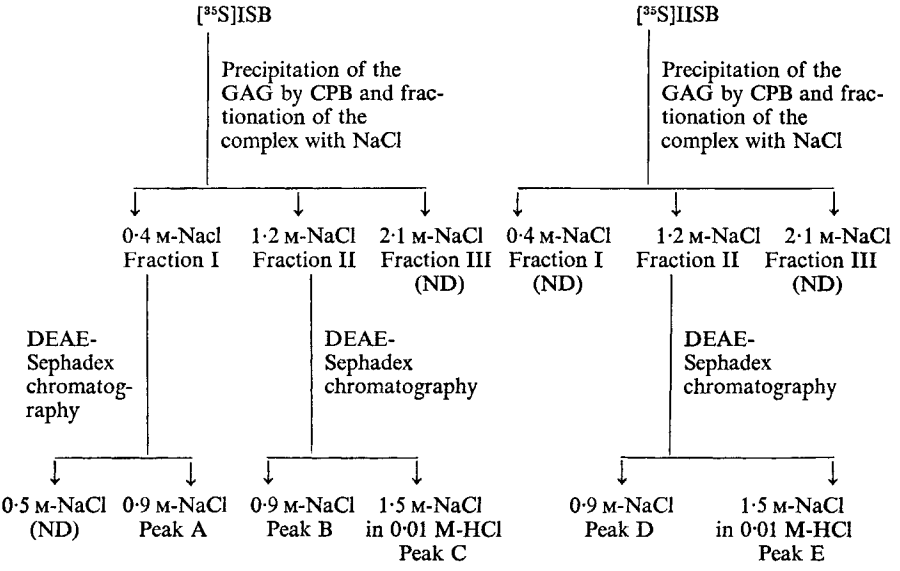
TABLE 2.—CPB FRACTIONATION OF LOW-SULPHATED GAG AFTER ENZYMIC SULPHATION

Sulphated GAG	Counts/min	
	Fraction I (0·4 M-NaCl extract)	Fraction II (1·2 M-NaCl extract)
[^{35}S]ISB	9400	11,700
[^{35}S]IISB	0	18,300
Hyaluronidase resistant		
[^{35}S]ISB	9600	10,700
[^{35}S]IISB	0	35,700

Carrier hyaluronic acid and chondroitin sulphate (1 mg each) and chondroitin sulphate (1 mg) were added to [^{35}S]ISB and [^{35}S]IISB respectively. After precipitation with CPB, the sediments were then extracted with various concentrations of NaCl. NaCl extraction was repeated until the extracts were free of radioactivity.

Hyaluronidase resistant fractions also incorporated 0·28-0·52 nmole of sulphate in the presence of the sulphotransferase system. There was no significant difference in the incorporation of [^{35}S]sulphate into the peptide free fractions and into the peptide rich fractions except in the case of hyaluronidase resistant ISB.

Characterization of [^{35}S]ISB *and* [^{35}S]IISB. The steps involved in the characterization of [^{35}S]ISB and [^{35}S]IISB after enzymic sulphation are summarized in the following scheme:



ND, Radioactivity not detectable.

The results of the fractionation of the [^{35}S]GAG-cetylpyridinium bromide complex with various concentrations of NaCl are shown in Table 2. The radioactivity of [^{35}S]ISB and hyaluronidase-resistant [^{35}S]ISB was equally distributed in both 0·4 M-NaCl and 1·2 M-NaCl fractions, whereas the radioactivity of [^{35}S]IISB and hyaluronidase-resistant [^{35}S]IISB was obtained in the 1·2 M-NaCl fraction only. All these fractions of [^{35}S]GAG obtained by the CPB procedure were further fractionated on DEAE-Sephadex. The 0·4 M-NaCl fraction of [^{35}S]ISB was completely eluted in one peak, A, as shown in Fig. 1, whereas the 1·2 M-NaCl fraction of [^{35}S]ISB was eluted in two peaks, B and C, showing that the charge density of the molecule had been increased. The elution pattern of peaks A, B and C were identical with the original glycosaminoglycan fractions ISB, IISB and IISC, respectively (SINGH and BACHHAWAT, 1968). Figure 2 shows that after *in vitro* sulphation of IISB, two radioactivity peaks, D and E, were obtained. The emergence of these peaks on sulphation of ISB and IISB indicated that ISB and IISB contained some lower-sulphated form of heparan sulphate, chondroitin-4-sulphate and chondroitin-6-sulphate.

Results of nitrous acid treatment of all the [^{35}S]GAG obtained after sulphation *in vitro* is given in Table 3. A typical gel-filtration profile of one of the fractions treated with nitrous acid is given in Fig. 3. Various radioactive peaks which were retained on Sephadex G-25 may be due to ^{35}S-sulphated oligosaccharides of varying

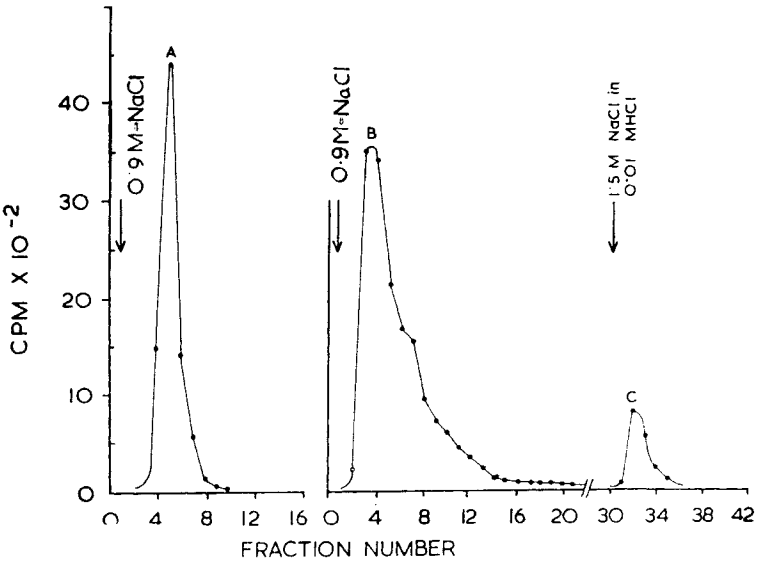


FIG. 1.—Elution pattern of [^{35}S]ISB on DEAE-Sephadex. After CPB fractionation, fraction I of [^{35}S]ISB (9400 counts/min) was applied to the column (8 × 0·9 cm) and eluted with 0·9 M-NaCl. Similarly, fraction II of [^{35}S]ISB (18,000 counts/min) was applied to the column and eluted with 0·9 M-NaCl and 1·5 M-NaCl in 0·01 N-HCl. Two ml fractions were collected and radioactivity was measured. CPM, Counts/min.

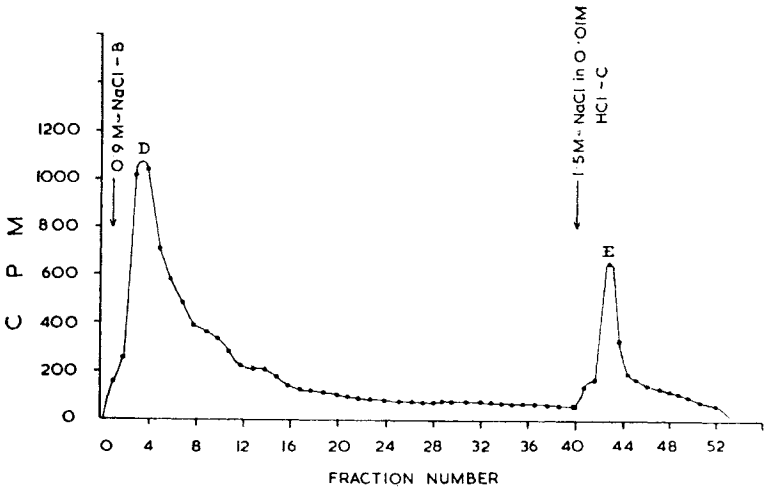


FIG. 2.—Elution pattern of[^{35}S]IISB on DEAE-Sephadex. The elution was carried out as for fraction II of [^{35}S]ISB except that [^{35}S]IISB (8500 counts/min) was applied to the column. CPM, Counts/min.

TABLE 3.—ANALYSIS OF [^{35}S]GAG FRACTIONS OBTAINED BY GEL FILTRATION AFTER NITROUS ACID DEGRADATION

Sulphated GAG	Radioactivity obtained (counts/min)	
	In void volume*	In inner volume†
[^{35}S]ISB	580	1090
Peptide-free [^{35}S]ISB	290	1060
[^{35}S]IISB	430	620
Peptide-free [^{35}S]IISB	590	640
Hyaluronidase resistant		
[^{35}S]ISB	650	1310
Peptide-free [^{35}S]ISB	1600	4860
[^{35}S]IISB	1140	1430
Peptide-free [^{35}S]IISB	750	1310

* [^{35}S]GAG which was unaffected by nitrous acid treatment was eluted in void volume.

† [^{35}S]Sulphate fractions containing oligosaccharides were retarded in the column.

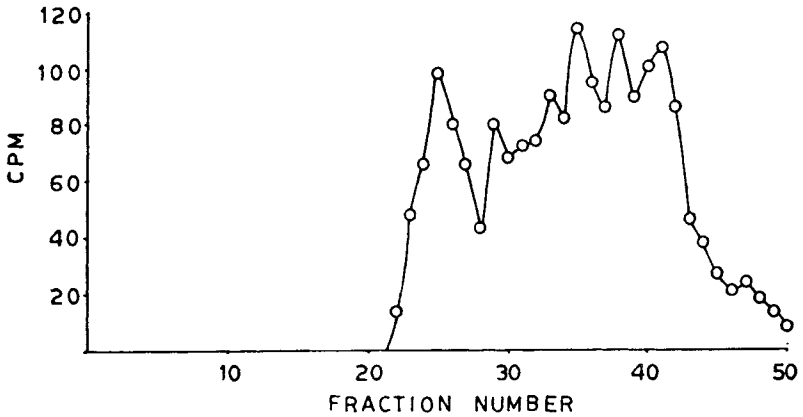


FIG. 3.—Elution pattern of the gel filtration of [^{35}S]GAG after nitrous acid degradation. CPM, counts/min. Experimental details are given in the text. One ml fractions were collected.

chain length. Furthermore, following *in vitro* sulphation, it was observed that when various GAG fractions were subjected to similar gel filtration without nitrous acid treatment, all the glycosaminoglycans were eluted in the void volume. A similar observation was made by CIFONELLI (1968) when he subjected heparan sulphate to gel filtration before and after nitrous acid treatment. All the [^{35}S]GAG fractions were partially degraded by nitrous acid treatment indicating that they contain heparan sulphate (*N*-sulphated GAG). The site of [^{35}S]sulphate incorporation into the low-sulphated GAG was further studied by 0·04 N-HCl hydrolysis. The results of HCl hydrolysis of low-sulphated GAG after enzymic sulphation is given in Table 4. A typical gel filtration pattern of [^{35}S]GAG after HCl hydrolysis is depicted in Fig. 4. The sulphamino group was easily removed under this condition of hydrolysis whereas only 20 per cent of the [^{35}S]sulphate of chondroitin-4-sulphate was removed. This result shows that both *N*- and *O*-sulphation were taking place in the molecule.

TABLE 4.—HCl HYDROLYSIS OF LOW-SULPHATED GAG AFTER SULPHATION *in vitro*

Sulphated GAG	Total products after hydrolysis (counts/min)	
	[^{35}S]GAG	Inorganic [^{35}S]sulphate
[^{35}S]ISB	17,000	9200
[^{35}S]IISB	8900	7700
Hyaluronidase resistant		
[^{35}S]ISB	12,700	13,800
[^{35}S]IISB	9200	14,800

Conditions of hydrolysis are given under Methods. After hydrolysis, gel filtration was carried out. The fraction obtained in void volume is designated [^{35}S]GAG and the fraction retarded in the column is inorganic [^{35}S]sulphate.

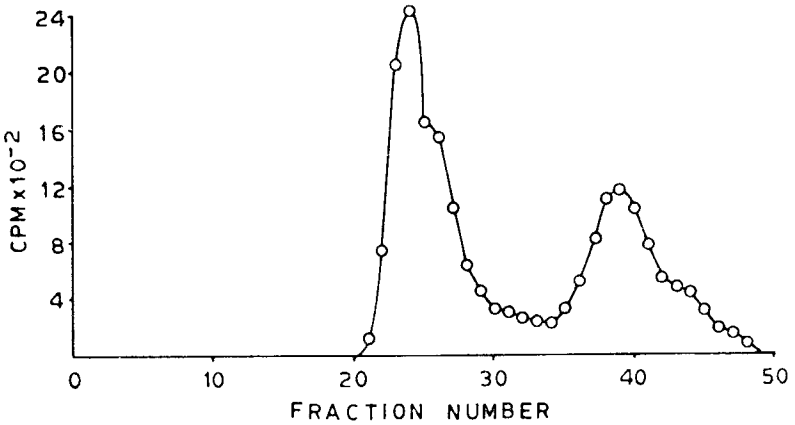


FIG. 4.—Elution pattern of the gel filtration of [^{35}S]GAG after HCl hydrolysis. CPM, Counts/min. Experimental details are given in the text. One ml fractions were collected.

When [^{35}S]ISB and [^{35}S]IISB were subjected to paper electrophoresis after *in vitro* sulphation, the relative mobilities of the two were increased as compared with the authentic ISB and IISB. The relative mobility of [^{35}S]ISB as compared with the standard chondroitin sulphate and heparan sulphate mixture was 0·85 whereas it was 0·81 before enzymic sulphation (SINGH and BACHHAWAT, 1968). Similarly the relative mobility of [^{35}S]IISB was 0·94 whereas before *in vitro* sulphation it was 0·85, indicating that there was an increase in anionic charge in the molecule.

Properties of neonatal human brain sulphotransferase. Since this crude sulphotransferase system catalysed both *N*- and *O*-ester sulphation, an attempt was made to purify sulphotransferase from autopsy samples of neonatal human brain. A 2·5-fold purified enzyme with 80 per cent yield was obtained by 45–65 per cent ammonium sulphate precipitation using chondroitin-4-sulphate as acceptor. The enzyme had maximum activity with phosphate buffer pH 7·4. It may be mentioned here that the sulphotransferase activity was very unstable. If the tissue was frozen, prior to extraction of the enzyme, complete loss of activity was observed within 24 hr. However, the 45–65 per cent ammonium sulphate fraction was stable for 2 weeks at −18°. It was observed that the 45–65 per cent fraction had a higher specific activity towards

chondroitin-4-sulphate and dermatan sulphate as compared to the 0–45 per cent fraction, whereas the specific activity towards heparan sulphate was high in the 0–45 per cent fraction as compared to that of the 45–65 per cent ammonium sulphate fraction. Following enzymic sulphation of heparan sulphate by both these fractions, the analysis of *N*- and *O*-sulphate by HCl hydrolysis showed that the *N*-sulphate to *O*-sulphate ratio was same in both the fractions.

Sulphation of various GAG acceptors using sulphotransferases from human brains of various ages. Sulphotransferase activity in child brain of various age-groups was studied in the 45–65 per cent ammonium sulphate fraction. [^{35}S]Sulphate incorporation into various glycosaminologlycans using brain sulphotransferase from these four age-groups are given in Table 5. The best acceptor in all cases was heparan sulphate,

TABLE 5.—SULPHOTRANSFERASE ACTIVITY OF HUMAN BRAIN OF DIFFERENT AGES TOWARDS VARIOUS GAG ACCEPTORS

GAG Acceptors	Enzyme activity*			
	1	2	3	4
Chondroitin-4-sulphate	4000	4490	4500	6000
Dermatan sulphate	9900	9000	7100	10,100
Chondroitin-6-sulphate	1800	N.D.	1600	840
N-Desulphoheparin	5700	7800	2300	3500
Heparan sulphate	34,800	28,000	24,000	19,300

* Enzyme activity is expressed as counts/min of [^{35}S]GAG formed per mg of enzyme protein under the conditions of assay.

Sulphotransferase (45–65% ammonium sulphate fraction) was prepared from: 1, premature baby brain; 2, 1-day-old child brain; 3, 7-year-old child brain; 4, 13-year-old child brain. Conditions of assay were the same as described in text. N.D., not determined.

followed by dermatan sulphate. *N*-Desulphoheparin was a poor acceptor, although 70 per cent of the amino group of *N*-desulphoheparin was free for sulphation (BALASUBRAMANIAN *et al.*, 1968). This shows that, in addition to free sites in the molecule available for sulphation, other pre-requisites are necessary for the sulphotransferase activity. Chondroitin-4-sulphate also accepted sulphate, the amount of incorporation being 4000–6000 counts/min/mg enzyme protein. Chondroitin-6-sulphate was a poor acceptor as compared to other GAG. There were variations in the enzymic sulphation of various GAG with the age of the brain. In general, sulphotransferase has a very high activity in neonatal brain towards all the GAG employed as acceptors. However, the specific activity of sulphotransferase towards heparan sulphate decreased as the age of tissue increased. The ratio of sulphotransferase activity towards heparan sulphate to that towards chondroitin-4- sulphate also decreased with age, it was 8·5 in premature baby brain, and decreased to 3·2 in the brain of a 13-year-old child. This finding with sulphotransferase of human brain is of interest since a similar change in sulphotransferase activity towards heparan sulphate and chondroitin-4-sulphate was made earlier in this laboratory (BALASUBRAMANIAN and BACHHAWAT, 1964) with extracts of rat brain.

Enzyme activity from brain preparations of various animals. The specific activities of the sulphotransferases obtained from brain of various species towards GAG is given in Table 6. The best acceptor in all cases, was heparan sulphate,

TABLE 6.—SULPHOTRANSFERASE ACTIVITY OF BRAIN PREPARATIONS FROM VARIOUS ANIMALS

GAG Acceptors	Enzyme activity*			
	Rabbit	Guinea pig	Rat	Human
Chondroitin-4-sulphate	980	500	1730	4500
Chondroitin-6-sulphate	500	N.D.	600	N.D.
Dermatan sulphate	1720	2280	5550	9000
N-Desulphoheparan	6330	1950	4760	7800
Heparan sulphate	19,840	12,600	23,360	28,370

* Enzyme activity is expressed as counts/min of [^{35}S]GAG formed per mg enzyme protein under the conditions of assay.

Protamine sulphate supernatant obtained from 1-day-old brain was used as enzyme. Data on sulphotransferase of neonatal human brain was obtained from Table 6 for comparison only. Conditions of assay are the same as described in text. N.D., Not determined.

followed by dermatan sulphate. All the species had the same pattern of specificity towards various GAG employed as acceptors.

Nature of enzymic sulphation and its age dependence. Since a decreased enzymic sulphation of haparan sulphate was noticed as the age of the brain increased, it was interesting to determine the type of sulphation taking place *in vitro*. The product obtained after gel filtration was dialysed and hydrolysed in 0·04 N-HCl as described earlier. Data obtained after hydrolysis were presented in Table 7. The amount of

TABLE 7.—HCl HYDROLYSIS OF HEPARAN [^{35}S]SULPHATE OBTAINED AFTER *in vitro* SULPHATION USING SULPHOTRANSFERASE OF DIFFERENT AGES

	Counts/min		*N*-sulphate/*O*-sulphate ratio
	Heparan [^{35}S]sulphate	Inorganic [^{35}S]sulphate	
* Human brain			
Premature baby	2040	21,580	10·0
Neonatal brain	6600	38,400	6·0
7-year-old child	930	2780	3·0
13-year-old child	3280	6400	1·9
† Rat brain			
1-day-old	360	2150	6·0
10-days-old	1960	3680	1·9
Adult (2-months-old)	4130	2610	0·65

* 45–65% Ammonium sulphate fraction was used as enzyme source.
† Protamine sulphate supernatant was used as enzyme source.
The experimental procedure was the same as in Table 4.

N-sulphation by the sulphotransferase was decreased as the brain matured. The ratio of *N*- to *O*-sulphation was 10·0 in the presence of sulphotransferase from premature human brain and this ratio decreased to 6·0 for neonatal human brain and 1-day-old rat and rabbit brain. Similarly, the ratio was found to decrease to 1·9 in the case of sulphotransferase from the brains of a 13-year-old child and a 10-day-old rat. The ratio of *N*- to *O*-sulphation was further decreased to 0·65 for adult rat brain.

DISCUSSION

The data presented in this paper relating to the *in vitro* enzymic sulphation of low sulphated GAG fraction, and the behaviour of these fractions towards nitrous acid treatment and mild aid hydrolysis, conclusively establishes that heparan sulphate is present in normal human brain. This confirms our earlier report of the presence of heparan sulphate in brain (SINGH and BACHHAWAT, 1968), although previous investigators (CLAUSEN and HANSEN, 1963; SZABO and ROBOZ-EINSTEIN, 1962) and MARGOLIS (1967) were unable to detect the presence of heparan sulphate in brain from various species. In the present investigation, GAG fractions having various ratios of sulphate to uronic acid were used as acceptors for the sulphotransferase system and it was shown that GAG with the lowest sulphate content was the best acceptor. This finding is of interest in the light of the report of MEEZAN and DAVIDSON (1967*a*) who found that those polysaccharides with a uronic acid/sulphate ratio of about 1 were the best acceptors. Since fractions with the lowest sulphate content are most active as acceptors and since there is no probability for chain elongation during sulphation in the present system, it can be concluded that the additional sulphation occurs after the completion of the polysaccharide chain. The present finding that peptide-free GAG and peptide-bound GAG can incorporate sulphate equally well is consistent with the work of MEEZAN and DAVIDSON (1967*b*) where they showed that peptide-free GAG is not a pre-requisite for the sulphotrans ferase system.

The origin of the low-sulphated fractions in brain is not yet known. They may arise as intermediates during the formation of GAG or may be formed by desulphation of GAG, since there are reports indicating the de-*N*-sulphation of heparin (LLOYD, EMBERY, POWELL, CURTIS and DODGSON, 1966) as well as desulphation of chondroitin-4-sulphate (HELD and BUDDECKE, 1967), in mammalian systems.

The studies on the specificity of human brain sulphotransferase from the two ammonium sulphate fractions with respect to various GAG acceptors, suggest the possibility of the existence of different sulphotransferase systems for various GAG acceptors. For example, in the present investigation the sulphotransferase present in the 0–45 per cent ammonium sulphate fraction had a higher specific activity towards heparan sulphate whereas the sulphatransferase of the 45–65 per cent fraction had considerably higher activity towards chondroitin-4-sulphate.

The specificity of sulphotransferase obtained from human brain of different ages towards various acceptors may reflect the total amount of the acceptor GAG present in the brain tissue at various ages (SINGH and BACHHAWAT, 1968). For example, the high activity of sulphotransferase towards heparan sulphate in neonatal brain and the decrease in its activity as age increases is consistent with the finding that concentration of heparan sulphate was highest in foetal brain and lowest in adult brain. The same pattern was observed for the concentration of chondroitin-6-sulphate in brain and the sulphotransferase activity towards chondroitin-6-sulphate. The specific activity of sulphotransferase towards chondroitin-4-sulphate gradually increased with age. The concentration of chondroitin-4-sulphate increased from 65 per cent in child brain to 85 per cent in adult brain.

Hydrolysis of radioactive products obtained from heparan sulphate after *in vitro* sulphation gave evidence that the amino group is sulphated more rapidly and more extensively in the early stages of brain maturation. The decrease in *N*-sulphation of

heparan sulphate as the brain matures in an interesting finding. It agrees with our previous work on the sulphotransferase system, when lower specific activity towards heparan sulphate was found in adult rat brain as compared to young rat brain (BALASUBRAMANIAN and BACHHAWAT, 1964). Although the significance of the change in specific activity of sulphotransferase towards heparan sulphate in relation to brain function and maturation is not known, it may be mentioned here that abnormal metabolism of heparan sulphate leads to a marked abnormality in brain function, as in the case of a mucopolysaccharidosis, namely San Fillipo Syndrome (MCKUSICK, 1966).

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