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ISOLATION AND CHARACTERIZATION OF GLYCOSAMINOGLYCANS IN BRAIN OF DIFFERENT SPECIES

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Abstracts—The uronic acid-containing glycosaminoglycans present in the brains of rat, monkey, chicken, sheep and rabbit were isolated into various fractions by combining the cetyl pyridinium procedure and DEAE-Sephadex column chromatography. The analyses of the fractions show that hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate, heparan sulphate and a testicular hyaluronidase-resistant galactosamine-containing GAG are present in the brain of all the species studied. Hyaluronic acid is the major GAG (33–41 per cent). Chondroitin-4-sulphate (19–35 per cent), and heparan sulphate (11–19 per cent), are the next prominent GAGs, in all the species except chicken. The results indicate the similarity in the pattern of GAGs in the brain of all the species.

IN RECENT years, a number of reports have appeared regarding the nature of glycosaminoglycans (GAGs) in the central nervous system (MEYER, HOFFMAN, GRUMBACH and SAMPSON, 1959; SZABO and ROBOZ-EINSTEIN, 1962; CLAUSEN and HANSEN, 1963; SINGH and BACHHAWAT, 1965; MARGOLIS, 1967). The variation in the pattern of GAGs in different species was indicated by the work of ONODERA, HIRANO, HORIUCHI and KASHIMURA (1966) who showed that bovine brain contains hyaluronic acid, chondroitin-4-sulphate and chondroitin-6-sulphate and that monkey brain contains hyaluronic acid and chondroitin-6-sulphate whereas chondroitin-4-sulphate and chondroitin-6-sulphate are present in the brain of pig and rabbit respectively. The work of MARGOLIS (1967) showed the presence of hyaluronic acid, chondroitin-4-sulphate and chondroitin-6-sulphate in bovine whole brain and the absence of GAGs from bovine myelin. Recent work from this laboratory (SINGH and BACHHAWAT, 1968) on the GAGs of human brain indicated the presence of hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparan sulphate and low-sulphated GAGs. These studies, which employed different procedures for the isolation of GAGs, indicate the variation in the pattern of GAGs in different species. In the present investigation, the nature and level of uronic acid-containing GAGs in the brain of the different species rat, monkey, man, chicken, sheep and rabbit are reported by employing the cetylpyridinium procedure and then DEAE-Sephadex chromatography for the isolation of GAGs.

MATERIALS AND METHODS

Glucosamine-HCl, galactosamine-HCl, hyaluronic acid and testicular hyaluronidase type I were obtained from Sigma Chemicals, U.S.A., chondroitin-6-sulphate from Miles Research Laboratory, U.S.A. and cetyl pyridinium bromide from Mann Research Laboratories. Sephadex G-25 and DEAE-Sephadex A-25 were supplied by Pharmacia Uppsala, Sweden. Papain (crude) was a gift from The Central Food Technological Research Institute, Mysore, India, and pronase B was obtained from California Biochemical Corporation, U.S.A. All the chemicals used were of analytical grade.

Abbreviation used: GAG, glycosaminoglycan.

The preparation of lipid-free brain and the isolation of glycosaminoglycans were performed as described earlier (SINGH and BACHHAWAT, 1968).

Constituent analysis. Uronic acid was determined by the carbazole reaction (DISCHE, 1947) as modified by BITTER and MUIR (1962) using glucuronolactone as the standard. For the identification of dermatan sulphate, the carbazole reaction was carried out with and without borate since iduronic acid gives a very low colour yield without borate in the reaction mixture. For the analysis of hexosamine the GAG fractions were hydrolysed in 6N-HCl in sealed tubes for 4 hr at 100° and the hydrolysates were dried *in vacuo* in presence of NaOH pellets and the residues were dissolved in water. Small samples were subjected to descending paper chromatography according to the method of MUKHERJI and SRI RAM (1964). The hexosamines were identified by the method of TREVELYAN, PROCTOR and HARRISON (1950). Total hexosamine was assayed by the method of GATT and BERMAN (1966) and galactosamine in presence of glucosamine was estimated by the procedure of LUDOWEIG and BENMAMAN (1967). Sulphate was estimated by the method of DODGSON and PRICE (1962) and non-acetylated hexosamine (*N*-sulphate) by the method of LAGUNOFF and WARREN (1962).

Hyaluronidase digestion and estimation of chondroitin-6-sulphate. The digestion was performed by the method of MATHEW and INOYUE (1961) and the estimation of tetrasaccharides containing *N*-acetyl amino sugar by the procedure of REISSIG, STROMINGER and LELOIR (1955) with known hyaluronic acid and chondroitin-6-sulphate as standards. For the determination of undigestible GAGs, the hyaluronidase digests were treated with cold TCA to 10% final concentration, the TCA supernatants were dialysed exhaustively against running tap water and then several changes of distilled water and the uronic acid contents were determined.

RESULTS

GAG-fractions in different species. The levels of the three GAG-fractions (obtained by the cetyl pyridinium procedure) in the brain of different species are given in Table 1. Compared to the human brain, rat and monkey brains had higher concentrations of

TABLE 1.—LEVELS OF GAG-FRACTIONS IN THE BRAINS OF DIFFERENT SPECIES

Species	μg Uronic acid/g dry lipid-free tissue				I/II
	Fraction I	Fraction II	Fraction III	Total	
Rat	1570	1830	15	3415	0·86
Monkey	1550	1320	2	2872	1·18
Man*	612	798	75	1485	0·78
Chicken	285	291	17	593	0·98
Sheep	280	220	65	565	1·27
Rabbit	177	300	Trace	477	0·59

* For comparison, these values were taken from an earlier paper (SINGH and BACHHAWAT, 19 68)

GAGs whereas chicken, sheep and rabbit brains had lower levels of GAGs. In all species, fractions I and II were predominant whereas fraction III was very low. When the levels of fractions I (predominantly non-sulphated GAGs) and II (sulphated GAGs) are compared, fraction II is higher than fraction I in the brain of rat, man and rabbit whereas this is reversed in monkey and sheep. The results of further resolution of fractions I and II by DEAE-Sephadex chromatography are presented in Table 2. In all species except sheep, fraction I-SA (hyaluronic acid) was five to 7·5 times the level of fraction I-SB and fraction II-SC (predominantly chondroitin sulphate) was higher than fraction II-SB (mainly heparan sulphate). These isolated GAG-fractions were subjected to various analyses. The analytical data are presented in Table 3.

Fraction I-SA. From paper chromatography and estimation of hexosamines, this fraction from all species was found to consist of glucosamine in equimolar ratio to uronic acid and a small amount of galactosamine in some species. Non-acetylated

TABLE 2.—DEAE-SEPHADEX CHROMATOGRAPHY OF THE GAG-FRACTIONS AND THE LEVELS OF THE SUBFRACTIONS IN THE BRAINS OF DIFFERENT SPECIES

Species	μg Uronic acid/g dry lipid-free tissue				I-SA / I-SB	II-SB / II-SC
	I-SA	I-SB	II-SB	II-SC		
Rat	1368	202	445	1385	6·70	0·320
Monkey	1368	182	528	792	7·29	0·660
Man*	528	84	296	502	6·25	0·590
Chicken	236	49	109	182	4·86	0·606
Sheep	205	75	110	110	2·73	1·000
Rabbit	156	21	88	212	7·56	0·415

* These values were taken from earlier paper (SINGH and BACHHAWAT, 1968).

TABLE 3.—ANALYTICAL DATA OF THE ISOLATED GAG-FRACTIONS

Species and GAG-fractions	Expressed as molar ratio of uronic acid				Hyaluronidase digestibility
	Hexosamine	Galactosamine / Glucosamine	Nonacetylated amino sugar	Sulphate	
Rat					
I-SA	0·98	0,195	0·028	0·159	100
I-SB	1·35	0·45	0·314	N.D.	N.D.
II-SB	0·86	0·69	0·316	1·07	51
II-SC	0·79	Galactosamine	0·039	0·89	67
Monkey					
I-SA	1·05	0·17	0·013	0·075	100
I-SB	1·13	0·30	0·197	0·95	N.D.
II-SB	1·19	1·09	0·186	0·48	50
II-SC	0·93	Galactosamine	0·037	1·46	63
Chicken					
I-SA	0·94	Glucosamine	0·019	0·075	100
I-SB	1·31	0·47	0·520	0·625	N.D.
II-SB	1·32	0·41	0·274	1·08	26
II-SC	1·02	Galactosamine	0·065	1·03	50
Sheep					
I-SA	1·03	0·17	0·009	Nil	100
I-SB	0·71	0·24	0·118	0·905	67
II-SB	0·77	0·80	0·121	0·885	49
II-SC	0·70	Galactosamine	0·015	0·900	82
Rabbit					
I-SA	0·86	Glucosamine	0·013	Nil	100
I-SB	0·86	Glucosamine	0·310	N.D.	N.D.
II-SB	1·05	0·66	0·306	0·93	N.D.
II-SC	0·96	Galactosamine	0·047	1·08	N.D.

N.D.—Not done because of insufficient material.

amino sugar was present in trace amounts and sulphate was either absent or very low. In all species, this fraction was completely digestible by hyaluronidase. These results confirm that this fraction consisted of hyaluronic acid in all species with a little chondroitin in the brain of rat, monkey and sheep.

Fraction I-*SB*. This fraction from all species had a considerable amount of non-acetylated amino sugar and sulphate. It consisted of both glucosamine and galactosamine, except in rabbit where only glucosamine was detected. This fraction from rat, man and chicken had 60–70 per cent glucosamine whereas that from monkey and sheep had more than 80 per cent glucosamine. In addition to the presence of non-acetylated amino sugar, the hyaluronidase digestibility determined for this fraction obtained from man and sheep showed that this fraction was only partially digestible. The results indicate that this fraction was a mixture of heparan sulphate and chondroitin sulphate (low-sulphated).

Fraction II-*SB*. This fraction was also a mixture of glucosamine-containing and galactosamine-containing GAGs. It contained considerable amounts of sulphate and non-acetylated amino sugar. Hyaluronidase digestion showed that the resistant GAGs in chicken brain were 74 per cent and in other species 30–50 per cent; chondroitin-6-sulphate and chondroitin-4-sulphate were present in this fraction (Table 4). The value for chondroitin-6-sulphate would include low-sulphated chondroitin-4-sulphate also because that also would give colour in the estimation of tetrasaccharides containing *N*-acetylated amino sugar (SINGH and BACHHAWAT, 1968).

TABLE 4.—RESULTS OF THE HYALURONIDASE DIGESTION OF FRACTIONS II-SB AND II-SC

Species	Fraction II-SB			Fraction II-SC		
	Hyaluronidase resistant	*Chondroitin-6-sulphate	Chondroitin-4-sulphate	Hyaluronidase resistant	Chondroitin-6-sulphate	Chondroitin-4-sulphate
Rat	49	40	1	33	9	58
Monkey	50	18	32	37	17	46
Chicken	74	18	8	50	33	17
Sheep	51	11	38	18	8	74
Rabbit	N.D.	25	N.D.	N.D.	26	N.D.

N.D.—Not done because of insufficient material.
* Would include low-sulphated chondroitin-4-sulphate that may be present in this fraction.

Fraction II-*SC*. This consisted of galactosamine-containing GAG. Hyaluronidase digestion shows that this fraction had considerable amounts of resistant GAG but the carbazole reaction without borate indicates that it may not be dermatan sulphate. The analyses showed that this fraction consisted of chondroitin-4-sulphate, chondroitin-6-sulphate and hyaluronidase-resistant galactosamine-containing GAG (Table 4). Chondroitin-4-sulphate was higher than chondroitin-6-sulphate in the brain of rat, monkey, and man. Human brain had very little resistant GAG whereas it was present in considerable amount in the other species.

The nature and quantity of the glycosaminoglycans in the various fractions are given in Table 5. Hyaluronic acid is the major GAG in all the species, studied. The next prominent GAG is chondroitin-4-sulphate in the brain of rat, monkey, man and sheep. In chicken brain chondroitin-6-sulphate is higher than chondroitin-4-sulphate and the level of heparan sulphate is also high compared to other species.

DISCUSSION

The procedure used for the isolation of GAGs by combining the cetyl pyridinium procedure and the DEAE-Sephadex column chromatographic method yielded pure

TABLE 5.—THE NATURE AND LEVEL OF THE VARIOUS GAGS IN THE DEAE-SEPHADEX FRACTIONS

Fractions	μg Uronic acid/g dry lipid-free brain					
	Rat	Monkey	Man*	Chicken	Sheep	Rabbit
I-*SA*						
Hyaluronic acid	1135	1176	528	236	208	156
I-*SB*						
Heparan sulphate	139	73	66	33	25	13
Galactosamine-GAG	63	42	18	16	50	—
II-*SB*						
Heparan sulphate	262	253	157	77	56	52
Chondroitin-6-sulphate	183	95	62	20	12	23
Chondroitin-4-sulphate	—	180	77	12	42	13
II-*SC*						
Hyaluronidase-resistant galactosamine-GAG	455	292	6	91	20	N.D.
Chondroitin-6-sulphate	125	136	66	60	9	55
Chondroitin-4-sulphate	805	364	430	31	81	N.D.

N.D.—Not done due to insufficient material.

* For comparison purpose, the values were incorporated from earlier paper (SINGH and BACHHAWAT, 1968).

non-sulphated GAG (Fraction I-SA) and equimolar sulphated galactosamine-GAG (Fraction II-SC), but the other fractions I-SB and II-SB are mixtures of glucosamine-containing and galactosamine-containing GAGs. The present study indicates that the concentration of total GAGs in brain varies from one species to another. The level of GAGs in rat and monkey brain is twice as high as in human brain while in sheep and chicken it is less than half and in rabbit one-third of the human value.

The ratios of fraction I to fraction II from the different species vary from 0·59 to 1·27. Further, the ratios of fraction I-SA to fraction I-SB vary from 2·73 to 7·56, and of II-SB to II-SC from 0·32 to 1·00. These results appear to indicate variation in the pattern of GAGs in different species as suggested by ONODERA *et al.* (1966). But the analyses of the above fractions show that all types of GAGs are present in the brain of all the species studied. The present results are not consistent with those of ONODERA *et al.* (1966) who reported that hyaluronic acid and chondroitin-4-sulphate were not present in rabbit brain and chondroitin-4-sulphate was not found in monkey brain. Further, there is not much variation in the levels of hyaluronic acid, heparan sulphate and chondroitin sulphate among the species except that chondroitin-4-sulphate is very low in chicken brain and hyaluronidase-resistant galactosamine-containing GAG is present in trace amounts in human brain. CUSTOD and YOUNG (1968) reported that the ratio of fraction I to fraction II is 1·43 for cat brain and this value is comparable with the value obtained for sheep and monkey brains. Further these authors have observed a greater reduction in fraction II than fraction I following the administration of hyaluronidase. This is explainable because fraction I is a mixture of hyaluronic acid and heparan sulphate. Further the amino sugar analysis of fraction III in some species shows that it consists of galactosamine-containing GAG only indicating that it is not heparin.

The present study shows that hyaluronic acid is the major GAG in the species studied, chondroitin-4-sulphate (except in chicken) and heparan sulphate are the next prominent GAGs indicating the similarity of the pattern of GAGs in all species. The present observation that chondroitin-6-sulphate is higher than chondroitin-4-sulphate in the chicken brain is of interest because in the other animals studied chondroitin-4-sulphate is much higher than chondroitin-6-sulphate. In a recent short communication, CUNNINGHAM and GOLDBERG (1968) report the presence of all types of GAGs in various species and this is consistent with the results of the present study. However, these authors have not given any quantitative data.

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REFERENCES

BITTER T. and MUIR H. (1962) *Analyt. Biochem.* **4,** 330.
CLAUSEN J. and HANSEN A. (1963) *J. Neurochem.* **10,** 165.
CUNNINGHAM W. L. and GOLDBERG J. M. (1968) *Biochem. J.* **110,** 35P.
CUSTOD J. T. and YOUNG I. J. (1968) *J. Neurochem.* **15,** 809.
DISCHE Z. (1947) *J. biol. Chem.* **167,** 189.
DODGSON K. S. and PRICE R. G. (1962) *Biochem. J.* **84,** 106.
GATT R. and BERMAN E. R. (1966) *Analyt. Biochem.* **15,** 167.
LAGUNOFF D. and WARREN G. (1962) *Arch. Biochem.* **99,** 396.
LUDOWIEG J. and BENMAMAN J. D. (1967) *Analyt. Biochem.* **19,** 80.
MARGOLIS R. U. (1967) *Biochim. biophys. Acta* **141,** 91.
MATHEW M. B. and INOYUE M. (1961) *Biochim. biophys. Acta* **53,** 509.
MEYER K., HOFFMAN P., GRUMBACH M. M. and SAMPSON P. (1959) *Proc. Soc. exp. Biol. (N.Y.)* **102,** 587.
MUKHERJI H. and SRI RAM J. (1964) *Analyt. Biochem.* **8,** 393.
ONODERA K., HIRANO S., HIRIUCHI F. and KASHIMURA N. (1966) *Carbohydrate Res.* **3,** 234.
REISSIG J., STROMINGER J. L. and LELOIR J. E. (1955) *J. biol. Chem.* **217,** 959.
SINGH M. and BACHHAWAT B. K. (1965) *J. Neurochem.* **12,** 519.
SINGH M. and BACHHAWAT B. K. (1968) *J. Neurochem.* **15,** 249.
SZABO M. M. and ROBOZ-EINSTEIN E. (1962) *Arch. Biochem.* **98,** 406.
TREVELYAN W. E., PROCTOR D. P. and HARRISON J. S. (1950) *Nature (Lond.)* **166,** 444.