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ISOLATION AND CHARACTERIZATION OF GLYCOSAMINOGLYCANS FROM BRAIN OF CHILDREN WITH PROTEIN-CALORIE MALNUTRITION

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Abstract—The uronic acid containing glycosaminoglycans (GAGs) were isolated from the brains of 1-year-old and 4-year-old kwashiorkor children and characterised by constituent analyses. A marked reduction is the total GAG concentration of brain was noticed in both cases of kwashiorkor. In the 1-year-old kwashiorkor brain, hyaluronic acid is the most predominant GAG (73·5 per cent) whereas heparan sulphate, chondroitin sulphates and low sulphated chondroitin sulphate constituted less than 10 per cent. In the 4-year-old kwashiorkor brain, the proportion of hyaluronic acid was 27·5 per cent, low sulphated chondroitin sulphate 31·2 per cent, chondroitin sulphates 28·3 per cent and heparan sulphate 10 per cent. This marked reduction in the concentration as well as qualitative changes in GAG in protein-calorie malnutrition as compared to the normal is discussed in relation to brain function.

THE OCCURRENCE of various glycosaminoglycans (GAGs) in brain has been established by the work of several laboratories. Although the functional significance of GAGs in brain has yet to be clearly understood, several reports indicate their important role in the brain function. MARGOLIS (1967), by showing that only a small portion of GAG in brain is of connective tissue origin, suggested that GAGs may have a distinct role in brain as compared with other organs. Earlier study in this laboratory (SINGH and BACHHAWAT, 1968) indicated the variation in the levels of these compounds in brain with age, suggesting their role in the process of myelination and brain maturation, as postulated by BRANTE in 1958. The recent work from this laboratory (GEORGE, SINGH and BACHHAWAT, 1969) has shown that the GAGs-sulphotransferase preparations from rat and human brain of various age groups differ in the nature of sulphation process, the ratio of *N*-sulphation to *O*-sulphation decreasing with the maturation of brain. In the mucopolysaccharidosis, Sanfilippo syndrome, which is associated with severe mental retardation, the accumulation of heparan sulphate in brain has been established (GEORGE and BACHHAWAT, 1970). The important role of GAGs such as hyaluronic acid and chondroitin sulphate in brain function is evident from the experiment of CUSTOD and YOUNG (1968) who observed in adult cat, after lateral ventricular injection of testicular hyaluronidase, neurological impairment in addition to a reduction in the levels of hyaluronic acid and chondroitin sulphate. A recent study from this laboratory (VASAN and BACHHAWAT, 1971) shows a decrease in the concentration of hyaluronic acid and low sulphated GAGs in rat brain in experimental allergic encephalomyelitis. Considering the above reports and the findings that there is an impairment of the active sulphate synthesis in rat liver on a low protein diet (LEVI, GELLER, ROOT and WOLF, 1968) and high urinary excretion of

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Abbreviation used: GAG, glycosaminoglycan.

low-sulphated chondroitin sulphate in kwashiorkor (CHERIAN, CHANDRASEKARAN and BACHHAWAT, 1970), it would be of interest to study the nature of GAGs in brain in protein-calorie malnutrition. The present investigation reports on the isolation and characterization of glycosaminoglycans in brain of two children of different ages in protein-calorie malnutrition.

MATERIALS AND METHODS

Papain (crude) and pronase B were received respectively from Central Food Technological Research Institute, Mysore and California Biochemical Corporation, U.S.A. Cetyl pyridinium bromide was obtained from Mann Research Laboratory, U.S.A. DEAE-Sephadex was supplied by Pharmacia, Uppsala, Sweden and chondroitin-6-sulphate containing 10 per cent chondroitin-4-sulphate by Miles Research Laboratory, U.S.A. Sigma Chemical Company, U.S.A. supplied glucosamine-HCl, galactosamine-HCl, hyaluronic acid from human umbilical cord (Grade I) and testicular hyaluronidase Type IV. Other reagents of analytical grade.

The brains from 1-year-old and 4-year-old kwashiorkor children were obtained at autopsy. The 1-year-old patient (female) had a body weight of 3·3 kg and was oedematous, which is an indication of severe kwashiorkor. The other patient (male) had a body weight of 6·2 kg and developed oedema at later stage. The delipidation of the brains and the isolation of glycosaminoglycans were carried out as described earlier (SINGH and BACHHAWAT, 1968).

Analyses. The modification of the carbazole reaction (DISCHE, 1947) by BITTER and MUIR (1962) was used to estimate uronic acid. This reaction was run with and without borate to identify dermatan sulphate. The total hexosamine, galactosamine, sulphate, non-acetylated hexosamine (*N*-sulphate), chondroitin-6-sulphate and hyaluronidase resistant GAG were estimated as described earlier (CHANDRASEKARAN and BACHHAWAT, 1969). All the analysis were carried out in triplicate.

RESULTS

The levels of the three-GAG-fractions isolated by employing the cetylpyridinium procedure are given in Table 1. Uronic acid 0·91 mg/g of lipid free dry tissue is present in the 1-year-old kwashiorkor brain whereas it is only 0·53 mg/g in the 4-year-old brain. The level of GAG/lipid free dry tissue of both fractions I and II are correspondingly higher (by 70–73 per cent) in the 1-year-old kwashiorkor brain when compared to the other brain and hence the values of ratio I/II for both brains are similar. Fraction III is extremely low in both cases.

TABLE 1.—LEVELS OF VARIOUS GLYCOSAMINOGLYCAN FRACTIONS IN 1-YEAR-OLD AND 4-YEAR-OLD KWASHIORKOR BRAINS

Kwashiorkor brain	μg uronic acid/g lipid free dry tissue Fraction I	Fraction II	Fraction III	Total	Ratio I/II
1-year-old	542·5	348·4	14·1	905	1·56
4-year-old	310·7	206·8	16·1	533·6	1·50

Fractions I, II and III are GAG fractions obtained from cetyl pyridinium bromide-GAG complex by extracting with 0·4 M; 1·2 M- and 2·1 M-NaCl, respectively.

In Table 2, the levels of the DEAE-Sephadex fractions are shown. The concentration of Fraction I-A in both is around 12 times that of Fraction I-B. Next to Fraction I-A,

TABLE 2. LEVELS OF GLYCOSAMINOGLYCANS IN VARIOUS DEAE-SEPHADEX FRACTIONS FROM 1-YEAR-OLD AND 4-YEAR-OLD KWASHIORKOR BRAINS

Kwashiorkor brain	μg uronic acid/g lipid free dry tissue				Ratio	
	I-A	I-B	II-A	II-B	I-A/I-B	II-A/II-B
1-year-old	502·3	40·2	335	13·4	12·5	25·0
4-year-old	287·7	23·0	206·8	—	12·5	—

Fraction I was further subfractionated on DEAE-Sephadex column and fractions I-A and I-B were obtained by eluting with 0·5 M- and 0·9 M-NaCl. Similarly, Fraction II was subfractionated on DEAE-Sephadex column and Fractions II-A and II-B were obtained with 9·9 M-NaCl and 1·25 M-NaCl in 0·01 N-HCl.

Fraction II-A is predominant in both brains. Fraction II-B occurs in low concentration in 1-year-old kwashiorkor brain whereas it is absent in 4-year-old brain.

The analytical data of the various GAG fractions isolated from the kwashiorkor brains are reported in Table 3. Table 4 presents the analytical data of the hyaluronidase resistant GAG fractions.

Fraction I-A. This fraction from both brains consists of equimolar hexosamine with respect to uronic acid. The amount of galactosamine is low in the case of 1-year-old kwashiorkor brain whereas it is higher than that of glucosamine in the 4-year-old brain. Sulphate is present in this fraction from both brains and its concentration is

TABLE 3.—THE ANALYTICAL DATA OF THE VARIOUS GLYCOSAMINOGLYCAN FRACTIONS FROM KWASHIORKOR BRAINS

Age group and fraction	Hexasamines*	Galactosamine† / Glucosamine	Sulphate	As percentage total GAG in the fraction: Chondroitin-6-sulphate	As percentage total GAG in the fraction: Hyaluronidase resistant
Kwashiorkor brain (1-year-old):					
I-A	1·03	0·1	0·26	—	
I-B	0·66	Glucosamine	1·34	—	57
II-A	0·76	0·36	1·43	—	23
II-B	1·31	0·30	1·04		Nil
III	0·70	3·9	1·03	44·0	24
Kwashiorkor brain (4-year-old):					
I-A	1·05	1·39	0·44	—	
I-B	0·99	0·91	1·32	—	61
II-A	0·60	2·00	0·89	—	24
III	0·50	7·00	0·89	48·0	11

* Expressed as molar ratio of uronic acid.

† Estimation of glucosamine and galactosamine was carried out by the method described in the text.

TABLE 4.—ANALYTICAL DATA OF HYALURONIDASE RESISTANT GAG FRACTIONS

Fraction	Expressed as molar ratio of uronic acid		
	N-sulphate	Hexosamines	Galactosamine
Kwashiorkor brain (1-year-old):			
I-B-HR*	0·25	2·34	0·46
II-A-HR	0·20	0·88	0·13
Kwashiorkor brain (4-year-old):			
I-B-HR	0·34	2·32	0·32
II-A-HR	0·31	1·56	0·09

* HR, Hyaluronidase resistant.

higher in 4-year-old brain, but *N*-sulphate was found to be absent in both cases. This fraction from both brains was digested with testicular hyaluronidase and then subjected to Sephadex G-25 filtration with water as eluent. The analysis of hexosamine indicates that the void volume material contains most of the galactosamine-containing GAGs. The void volume GAG was precipitated by cetyl pyridinium bromide, extracted with 0·4 M-NaCl containing 0·1 per cent cetyl pyridihium bromide and then precipitated with 4 vol. of alcohol. This isolated GAG was subjected to the carbazole reaction with and without borate. Since the depression in colour was not as marked as in the case of dermatan sulphate, this galactosamine containing hyaluronidase resistant material, as yet remains to be identified.

Fraction I-B. In the case of 1-year-old kwashiorkor brain, 0·66 M-hexosamine with respect to uronic acid is present and in 4-year-old brain, the molar ratio of hexosamine to uronic acid is 0·99. The concentration of galactosamine is almost equal to that of glucosamine in 4-year-old brain whereas the amino sugar is absent in this fraction of the other brain. The sulphate content of both is 1·3 molar with respect to uronic acid. The hyaluronidase resistant material is 57 per cent of this fraction in 1-year-old kwashiorkor brain and 61 per cent in the 4-year-old brain. The hyaluronidase resistant fraction of 1-year-old kwashiorkor brain contains 0·25 M-N-sulphate with respect to uronic acid and 54 per cent glucosamine, indicating that all of the glucosamine GAG is heparan sulphate. The molar ratio of *N*-sulphate to uronic acid is 0·34 and 70 per cent glucosamine are present in the hyaluronidase resistant portion of 4-year-old brain, indicating again that the glucosamine GAG is heparan sulphate. The presence of low amount of galactosamine in this fraction from 1-year-old kwashiorkor brain may be the cause of the inability to identify it, when the whole fraction was subjected to hexosamine analysis, Further, the high total hexosamine values noticed in the hyaluronidase resistant fractions could not be explained.

Fraction II-A. The molar ratio of hexosamine to uronic acid is 0·76 and 0·60 respectively in the case of 1-year-old and 4-year-old kwashiorkor brains. The amount of galactosamine is twice and 0·36 times that of glucosamine and sulphate to uronic acid ratio is 0·89 and 1·43 in 4-year-old and 1-year-old kwashiorkor brains, respectively. In 1-year-old kwashiorkor brain, the hyaluronidase resistant material is 23 per cent; the resistant material contains 13·4 per cent galactosamine only and hence, the

resistant galactosamine GAG in the whole fraction is 3 per cent. The whole fraction consists of 26 per cent galactosamine indicating the presence of 26 per cent chondroitin sulphate. The hyaluronidase resistant material from 1-year-old kwashiorkor brain contains 86·6 per cent glucosamine and the molar ratio of *N*-sulphate to uronic acid is 0·2 suggesting the presence of heparan sulphate which is probably low *N*-sulphated. In the same way, 4-year-old brain contains 64·6 per cent chondroitin sulphate and 2·4 per cent hyaluronidase resistant galactosamine GAG. The hyaluronidase resistant material contains 90 per cent glucosamine and the molar ratio of *N*-sulphate to uronic acid is 0·3 indicating the occurrence of heparan sulphate which is probably low *N*-sulphated.

TABLE 5.—THE NATURE AND AMOUNT OF GLYCOSAMINOGLYCANS IDENTIFIED IN THE VARIOUS FRACTIONS ISOLATED FROM THE KWASHIORKOR BRAINS

Fraction	Nature of glycosaminoglycans	μg uronic acid/g lipid free dry tissue: 1-year-old kwashiorkor brain	4-year-old kwashiorkor brain
I-A:	Glucosamine-GAG (hyaluronic acid)	457	121
	Galactosamine-GAG (low sulphated chondroitin sulphate)	45·3	166·7
I-B:			
Hyaluronidase resistant	(a) Heparan sulphate	12·5	9·9
	(b) Galactosamine-GAG	10·5	4·1
Hyaluronidase digestible	(a) Glucosamine-GAG (hyaluronic acid)	17·2	2·1
	(b) Galactosamine-GAG *(chondroitin sulphate)	—	6·9
II-A:			
Hyaluronidase resistant	(a) Heparan sulphate (less *N*-sulphated)	66·7	44·7
	(b) Galactosamine-GAG	10·3	5·0
Hyaluronidase digestible	(a) Chondroitin sulphate	77·1	133·6
	(b) Glucosamine-GAG (hyaluronic acid)	180·9	23·5
II-B:			
Hyaluronidase resistant		—	—
Hyaluronidase digestible	(a) Glucosamine-GAG (hyaluronic acid)	10·3	—
	(b) *Chondroitin sulphate	3·1	—
III:			
Hyaluronidase resistant		3·4	1·8
Hyaluronidase digestible	(a) Chondroitin-6-sulphate	6·2	7·7
	(b) Chondroitin-4-sulphate	1·7	4·7

* Not analysed for chondroitin-6-sulphate due to the presence of hyaluronic acid in these fractions.

Fraction II-B. It is absent in 4-year-old brain. In 1-year-old kwashiorkor brain, this fraction had molar ratio of hexosamine to uronic acid of 1·3 out of which 23 per cent is galactosamine, the rest being glucosamine. Though the molar ratio of sulphate to uronic acid is 1 the results that there is no hyaluronidase resistant GAG as well as *N*-sulphate, indicate that the glucosamine GAG must be hyaluronic acid.

Fraction III. In 1-year-old kwashiorkor brain galactosamine constitutes 80 per cent of the total hexosamine. Sulphate concentration is 1 molar with respect to uronic acid. The hyaluronidase resistant GAG and chondroitin-6-sulphate are 24 and 44 per cent respectively, chondroitin-4-sulphate being 12 per cent. The molar ratio of hexosamine to uronic acid is 0·5 mg in 4-year-old brain; 88 per cent of the hexosamine is galactosamine and molar ratio of sulphate to uronic acid is 0·89; chondroitin-6-sulphate and hyaluronidase resistant GAG are 48 and 11 per cent, respectively, whereas chondroitin-4-sulphate is 29 per cent.

TABLE 6.—THE AMOUNT OF GLYCOSAMINOGLYCANS IDENTIFIED IN THE 1-YEAR-OLD AND 4-YEAR-OLD KWASHIORKOR BRAINS

Glycosaminoglycans	Concentration in brain as the percentage of total GAGs	
	1-year-old kwashiorkor brain	4-year-old kwashiorkor brain
Hyaluronic acid	73·5	27·5
Heparan sulphate	8·8	10·2
Hyaluronidase resistant, galactosamine-GAG	2·3	1·7
Low sulphated chondroitin sulphate	5·0	31·2
Chondroitin-sulphate*	8·9	26·4
Chondroitin-4-sulphate	0·7	1·4
Chondroitin-6-sulphate	0·1	0·9

* Not analysed for chondroitin-6-sulphate due to the presence of hyaluronic acid in these fractions.

The nature and amount of glycosaminoglycans in each fraction are presented in Table 5. The concentrations of the various GAGs identified in the brains of kwashiorkor children are expressed as the percentage of the total GAGs in Table 6. In the brain of 1-year-old kwashiorkor child, hyaluronic acid is the major GAG (73·5 per cent) whereas the other GAGs such as heparan sulphate, chondroitin-4 and 6-sulphates are less than 10 per cent. On the other hand, low sulphated chondroitin sulphate is the major GAG (31·2 per cent) and hyaluronic acid and chondroitin sulphate are the next prominant GAGs in 4-year-old kwashiorkor brain.

DISCUSSION

The earlier work from this laboratory has shown the concentration of GAGs in the brain of 1-year-old normal child as 2·62 mg uronic acid/g lipid free dry tissue. The present investigation shows the presence of 0·91 mg and 0·53 mg uronic acid/g of dry defatted tissue in the brains of 1-year-old and 4-year-old kwashiorkor children, respectively. This indicates that there is a marked reduction in the GAG concentration in brain due to protein calorie malnutrition.

The results of the present study show that hyaluronic acid (73·5 per cent of total GAGs) is the major GAG in 1-year-old kwashiorkor brain whereas it is only one of the predominent GAGs in 4-year-old brain. In the brain of normal child, hyaluronic acid was found to be 36 per cent of the total GAGs. From these observations, it appears that the percentage of hyaluronic acid in total GAG has increased in brain due to kwashiorkor. On the other hand, if the concentration of hyaluronic acid is expressed as the amount/g lipid free dry tissue, the values are 0·67 mg, 0·15 mg and 0·94 mg, respectively, for the brains of 1-year-old and 4-year-old kwashiorkor and 1-year-old normal children indicating thus a considerable low level of hyaluronic acid in kwashiorkor brain. In this connection, it is to be noted that hyaluronic acid which is found in Fraction I-A only in normal brain, is occurring in other fractions also in kwashiorkor brain and this finding is not explainable.

Heparan sulphate has been shown earlier to constitute about 5 per cent of the total GAGs in 1-year-old normal child brain and it is 8·9 per cent in 1-year-old kwashiorkor brain and 10·2 per cent in 4-year-old brain. Although there is a percentage increase in heparan sulphate concentration, on the basis of concentration/g lipid free dry tissue, the level of heparan sulphate has not changed much as compared to normal brain.

In the previous investigation 10 per cent of the total GAGs in 1-year-old normal child brain has been found to be low sulphated chondroitin sulphate. In 1-year-old kwashiorkor brain, it is only 5 per cent, whereas it is 31·2 per cent in 4-year-old brain, thus indicating a high level of low sulphated chondroitin sulphate in the 4-year-old kwashiorkor brain; but on the basis of concentration/g lipid free dry tissue, this increase in 4-year-old kwashiorkor brain would appear to be not significant. On the other hand, when the levels of other GAGs/g of dry defatted tissue have correspondingly undergone reduction due to the reduction in the concentration of total GAGs, the present finding that the level of low sulphated chondroitin sulphate has increased in 4-year-old brain is definitely a significant one.

It has been reported earlier that in kwashiorkor, an increase in the urinary excretion of lysosomal arylsulphatase (ITTYERAH, DUMM and BACHHAWAT, 1967) and a decrease in the urinary excretion of bound sulphate (ITTYERAH, 1969) occur, indicating the labilization of lysosomes. In consistent with this observation, the subsequent investigation (CHERIAN *et al.*, 1970) has shown the presence of considerable amount of low sulphated chondroitin sulphate in urine of kwashiorkor children. The results of the present study that the level of chondroitin-4-sulphate is extremely low (0·7 per cent) in 1-year-old kwashiorkor brain when compared to that of 1-year-old normal brain (34 per cent), whereas the level of low sulphated chondroitin sulphate is high in 4-year-old kwashiorkor brain are consistent with the above findings.

The work of CLARK, NAISMITH and MUNRO (1957) has indicated that on a low protein diet, the rate of liver protein synthesis is lower than on a high protein diet. Assuming that the same change occurs in brain due to low protein diet, the low level of chondroitin sulphate in kwashiorkor brain may be due to the fact that the synthesis of the protein core is necessary for the elongation of the GAG chain and so an impairment in the synthesis of chondroitin sulphate would tend to occur in protein deficiency; the present finding that the level of hyaluronic acid in brain is reduced to a less extent in kwashiorkor when compared to chondroitin sulphate may be due to that hyaluronic acid is known to exist as free polyssaccharide in tissues.

LEVI *et al.* (1968) have shown that the level of ATP-sulphate adenylyl transferase in-

volved in the synthesis of active sulphate is less in liver on a low protein diet. Assuming a similar reduction in the level of that enzyme in brain on a low protein diet, the increase in the level of low sulphated chondroitin sulphate in 4-year-old brain is an interesting observation.

CUSTOD and YOUNG (1968) noticed in their experimental cat with limited administration of testicular hyaluronidase, a withdrawn and unresponsive behaviour. Patients with kwashiorkor in the advanced state exhibit considerable mental apathy. This remarkable similarity between kwashiorkor patients and experimental animal justifies at least to some extent the important role of GAGs in brain function.

The present study has thus indicated the changes in the levels of chondroitin sulphate and low sulphated chondroitin sulphate in brain in protein-calorie malnutrition.

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REFERENCES

BITTER T. and MUIR H. (1962) *Analyt. Biochem.* **4,** 330.
BRANTE G. (1959) in *Biochemistry of the Central Nervous System* (Edited by BRUCKE, F.) p. 291. Pergamon, New York.
CHANDRASEKARAN E. V. and BACHHAWAT B. K. (1969) *Biochim. biophys. Acta* **177,** 265.
CHERIAN R., CHANDRASEKARAN E. V. and BACHHAWAT B. K. (1970) *Indian J. Biochem.* **7,** 174.
CUSTOD J. T. and YOUNG I. J. (1968) *J. Neurochem.* **15,** 809.
CLARK C. M., NAISMITH D. J. and MUNRO H. N. (1957) *Biochim. biophys. Acta* **23,** 587.
DISCHE Z. (1947) *J. Biol. Chem.* **167,** 189.
GEORGE E. and BACHHAWAT B. K. (1970) *Clinica chim. Acta* **30,** 317.
GEORGE E., SINGH M. and BACHHAWAT B. K. (1969) *J. Neurochem.* **17,** 189.
ITTYERAH T. R., DUMM M. E. and BACHHAWAT B. K. (1967) *Clinica chim. Acta* **17,** 405.
ITTYERAH T. R. (1969) *Clinica chim. Acta* **25,** 365.
LEVI A. S., GELLER S., ROOT, D. M. and WOLF G. (1968) *Biochem. J.* **109,** 39.
MARGOLIS R. U. (1967) *Biochim. Biophys. Acta.* **141,** 91.
SINGH M. and BACHHAWAT B. K. (1968) *J. Neurochem.* **15,** 249.
VASAN N. S. and BACHHAWAT B. K. (1971) *J. Neurochem.* **18,** 1853.