Further studies on hepatic acidic glycosaminoglycans in the Hurler syndrome[1]

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Summary. Acid GAG were isolated from hepatic tissue from 3 patients with Hurler syndrome and 3 normal controls. Gross elevations in the uronic acid and hexosamine contents were found in Hurler livers compared with the normal ones. The total GAG concentration was significantly increased (about 25fold) in Hurler patients.

The Hurler syndrome (mucopolysaccharidosis type I) is a genetically determined disorder, which is characterized by excessive visceral storage and urinary excretion of heparitin sulfate and dermatan sulfate[3]. Faulty degradation of the above glycosaminoglycans (GAG) owing to a deficit of α-1-iduronidase appears to be the primary biochemical defect in the Hurler syndrome[4,5]. In addition to that, different authors have described the storage in liver and spleen of readily soluble, highly degraded glycosaminoglycans.[6,7].

This report presents data on the concentration of acidic glycosaminoglycans isolated from liver tissue from 3 patients with the Hurler syndrome and 3 normal controls, in order to get further insight into the biochemical changes in the Hurler syndrome.

Material and methods. The patients used in this study were 3 white boys 4 years and 4 months; 4 years and 8 months, and 4 years and 11 months old, respectively, affected by Hurler syndrome (mucopolisaccharidosis, type I). The diagnoses were established on the basis of family pedigrees, clinical and radiological findings and measurements of GAG on urine, plasma and cultured skin fibroblast and their media. The mental adequacy of the patients could not be properly evaluated owing to their young age.

In all Hurler patients, liver tissue was obtained at autopsy within 3 h of death. 3 clinically normal boys of 4 years and 2 months; 4 years and 6 months, and 4 years and 7 months old respectively were used as controls. Tissue samples were obtained by liver biopsy and stored as immediately frozen. Previous studies from this laboratory have shown that there is no significant difference between GAG in liver tissue obtained at autopsy when compared with that obtained from biopsy.

All samples were freed of blood and extraneous materials and frozen at $-22\,^{\circ}C$ until used. About 3 g of each liver were defatted and dehydrated for 36 h with 2 changes of ether-acetone (1:1 v/v) and were then dried at $80\,^{\circ}C$ for several hours until constant weight was reached; 1 g of the dried-defatted tissue was minced, homogenized in a Waring Blendor and suspended in 3 ml phosphate buffer, pH 7.5 and heated at $100\,^{\circ}C$ for 20 min. In 6 ml of the same buffer 16 mg of trypsin (EC 3.4.4.4. Nutritional Biochemicals Corp. Cleveland, USA) were heated at $37\,^{\circ}C$ and added to the tissue (1.5 ml per sample) and the mixture was incubated for an additional 24 h.

After digestion, trichloroacetic acid (TCA) was added until a final concentration of 5% was obtained. The precipitate was then washed with the same volume of TCA. 3 vol. of 5% potassium acetate in ethanol were then added to the combined supernatants.

After standing for 24 h at $3\,^{\circ}C$, the precipitated total crude glycosaminoglycans (GAG) were purified by dissolving them in 1.25 M magnesium chloride. The GAG were precipitated with 3 vol. of 2% sodium acetate in 95% ethanol during 24 h. Further purification was obtained by redissolving them in 5% potassium acetate and reprecipitating with 3 vol. of 95% ethanol for 12 h. Uronic acids were determined on an aliquot of the above, using the method of Bitter and Muir[8], and the original reaction described by Dische[9]. Total hexosamine was measured following the method of Cessi and Pillego[10] after hydrolysis of aliquot fractions in 4 N HCl at $100\,^{\circ}C$ for 4 h using glucosamine HCl as standard. Galactose was measured by the technique of Dische[11]. Total sialic acids by the technique of Warren[12] and total hexoses were measured with the method of Yemm and Willis[13]. The purified GAG were then fractionated by chromatography on cellulose microcolumns by the technique of Svejcar and Robertson[14]. In order to quantitate the different GAG, uronic acid concentration was also determined on each GAG fraction. Identity of each GAG fraction was tested by the characteristic column chromatography elution pattern and IR-spectra. Recoveries of 20–100 µg of chondroitin-4-sulfate alone, or when added to 50 mg of liver tissue and carried through the entire procedure, varied between 86–92%.

Results and discussion. Table 1 shows the chemical analysis of the crude GAG from normal and Hurler livers. Figures are expressed as mg/g±SEM of dry defatted tissue. Figures for uronic acids in both groups are quite similar when measured with the Bitter and Muir reaction and the orcinol method, hence most of the uronic acid should be D-glucuronic acid. Gross elevations in the uronic acid and hexosamine content were found in Hurler liver as compared with the normal ones. Traces of galactose were found only in Hurler liver. Total hexoses were increased (about 50%) and total sialic acids content was also increased (3fold increase).

Table 2 demonstrates the concentration of the GAG fractions in Hurler and control livers expressed in mg/g±SEM dry tissue.

Heparitin sulfate has been reported to contribute the bulk of the stored GAG in the liver of most of the patients with

Table 1. Chemical analysis of the crude glycosaminoglycans from normal and Hurler liver

	Normal liver	Hurler liver
Uronic acids		
Carbazol method	1.72 ± 0.15	43.8 ± 3.81
Orcinol method	1.66 ± 0.16	36.3 ± 2.09
Hexosamine	2.37 ± 0.26	34.8 ± 2.61
Galactose	–	1.12 ± 0.09
Total hexoses	11.7 ± 1.01	17.6 ± 1.28
Total sialic acids	0.16 ± 0.02	0.48 ± 0.04

Figures are expressed as mg/g±SEM of dry defatted tissue. Each figure represents the average concentration of 3 normal livers and 3 Hurler livers, respectively.

Table 2. Concentration of acidic glycosaminoglycans in normal and Hurler liver

	Normal liver	Hurler liver
Total GAG	4.35 ± 0.21	109.4 ± 7.63
Heparitin sulfate	3.53 ± 0.31	89.7 ± 8.21
Dermatan sulfate	0.98 ± 0.05	13.6 ± 1.28
Recovery (by addition)	4.51	103.3

GAG concentration was based on 40% uronic acid content. Figures are expressed as mg of GAG/g± SEM of dry defatted tissue.

mucopolysaccharidosis type I, the other component being dermatan sulfate[15]. In addition to that, Gordon et al.[16] have found that GAG were almost exclusively heparan sulfate in the mucopolysaccharidosis type III (Sanfilippo syndrome) and a mixture of heparitin and dermatan sulfate in the type I and II (Hurler and Hurler syndromes). The total GAG concentration was significantly increased (about 25fold) in our Hurler patients. In control livers, heparitin sulfate accounts for 81% of the total GAG content, the other component being dermatan sulfate (22%). In our Hurler patients, liver heparitin sulfate accounts for 82% of the total GAG, but dermatan sulfate content is about 12%. Compared to the uronic acid-containing compounds, changes in the glycoproteinic sugars are small. The most marked effects were obtained for total sialic acid (3fold increase) and total hexoses (50% increase). The above results confirm and extend those of Gordon[16] and Roukema[17] on the content and distribution of acid glycosaminoglycans and glycoproteins in the Hurler syndrome.

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2 Member of the 'Carrera del Investigador Científico', Conicet, Argentina.
3 N.K. Terry and A. Linker, Proc. Soc. exp. Biol. Med. *115*, 394 (1964).
4 R. Matalon, J.A. Cifonelli and A. Dorfman, Biochem. biophys. Res. Commun *42*, 340 (1971).
5 R. Matalon and A. Dorfman, Biochem. biophys. Res. Commun *47*, 959 (1972).
6 J. Knecht, J.A. Cifonelli and A. Dorfman, J. biol. Chem. *242*, 4652 (1967).
7 M.F. Dean, H. Muir and J.R.F. Ewins, Biochem. J. *123*, 883 (1971).
8 T. Bitter and H.M. Muir, Analyt. Biochem. *4*, 330 (1962).
9 Z. Dische, in: Methods in Biochemical Analysis, vol.2, p.313. Ed. D. Glick. Interscience New York, 1955.
10 A. Cessi and A. Pillego, Biochem. J. *77*, 508 (1960).
11 Z. Dische, in: Methods in Biochemical Analysis, vol.2, p.327. Ed. D. Glick. Interscience New York, 1955.
12 L. Warren, J. biol. Chem. *234*, 1971 (1959).
13 E.W. Yemm and A.J. Willis, Analyt. Biochem. *57*, 508 (1954).
14 J. Svejcar and W. van B. Robertson, Analyt. Biochem. *18*, 333 (1967).
15 K. Meyer and P. Hoffman, Arthritis Rheum. *4*, 552 (1961).
16 B.A. Gordon and M. Daria Haust, Clin. Biochem. *4*, 147 (1971).
17 P.A. Roukema, C.H. Oderkerik and G. Van den Berg, Clinica chim. Acta *44*, 277 (1973).

Depression by ethionine of phosphorylating oxidation in hepatic mitochondria

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Summary. Induction of hepatic steatosis and suppression of hepatic ATP levels, protein synthesis and gluconeogenesis subsequent to administration of ethionine may be consequences of interference by this compound with mitochondrial phosphorylation of ADP. The mitochondrial dysfunction is not a direct action of ethionine on the organelle.

Administration of ethionine results in a variety of morphological and biochemical lesions in the liver and other organs of the rat. Ethylation (presumably via S-adenosylethionine) of hepatic nucleic acids occurs[1] and a high incidence of hepatocellular carcinomas result[2]. The aminoacyl-tRNA synthetase does not descriminate between methionine and ethionine, and when the latter is fed it is incorporated into proteins in positions normally occupied by methionine[3]. Therefore, reduction of protein biosynthesis[4,5] is probably indirect and may be due to an energy deficit consequent to an effect on oxidative phosphorylation. It has been proposed that depression of protein biosynthesis occurs when ethionine, replacing methionine in S-adenosylmethionine, traps available adenosine and thereby prevents synthesis of ATP[6].

The ethionine induced fatty liver is preceded by a marked decrease in hepatic ATP levels[7], is prevented by administration of ATP[8], and is thought to be associated with, or a consequence of, inhibition of hepatic protein synthesis via a block in the synthesis of lipoprotein apoproteins for subsequent transport[9]. Ethionine injection also suppresses gluconeogenesis and hepatic ATP levels and results in increased free fatty acids in liver, plasma and adipose. These parameters return to normal simultaneously with recovery of the hepatic ATP concentration[10]. Moreover, ethionine itself is capable of inhibiting induction of fatty livers due to choline deficiency or orotic acid feeding[11] and this may be another reflection of depressed ATP availability and its requirement in triglyceride synthesis. Livers of rats treated with ethionine were found to have an altered ratio and

Table 1. In vitro effect of ethionine on mitochondrial respiratory activities

DL-ethionine (mM)	Glutamate respiration State 3	State 4	RCR	Succinate respiration State 3	State 4	RCR
0	52.2±4.9	10.7±0.3	4.87±0.28	132.9±7.5	24.7±0.7	5.36±0.18
10	52.3±4.1	11.2±0.4	4.68±0.34	129.8±9.3	26.9±1.0[f]	4.81±0.20[f]
20	51.0±4.1	12.4±0.7[e]	4.16±0.42	-	-	-
30	51.0±4.2	13.0±0.4[a]	3.99±0.45	135.2±7.6	28.4±0.7[b]	4.74±0.17[d]
100	54.1±3.4	14.0±0.4[a]	3.85±0.22[c]	139.8±5.6	31.4±0.6[a]	4.45±0.16[b]

Respiratory rates (ng atoms of oxygen consumed/min/mg mitochondrial protein) were determined polarographically at 30 °C with a Clark fixed voltage electrode. The 3 ml reaction mixture (pH 7.4) contained the indicated concentration of DL-ethionine plus 0.33 M mannitol, 5 mM $MgCl_2$, 3.5 mM potassium phosphate, 3.5 mM KCl, 0.33 mM EDTA, 4 mg dialyzed crystalline bovine serum albumin, 1.4 mM L-glutamate or succinate, and mitochondria corresponding to 2.5 mg of mitochondrial protein. RCR is the ratio of the respiratory velocity in presence of 0.4 µmoles ADP (added in 30 µl) (state 3) to the velocity after exhaustion of ADP (state 4). Means ± SE for 5 mitochondrial preparations at each ethionine concentration and 10 control preparations. [a]$p<0.001$; [b]$p<0.005$; [c]$p<0.02$; [d]$p<0.025$; [e]$p<0.05$; [f]$p<0.1$.