ception to occur from Day 8–10 and 7–9 respectively in cases of petroleum ether and chloroform extracts. Table II indicates that the basic part and the Fractions I and III of the acid part of the chloroform extract possess maximal interceptive activity which is less, though still significant, in the cases of the neutral part and the chromatographic fraction II of the acid part. The interception was complete also within the period of Day 7–9 in all these cases, except in that of the acidic fraction III which required a longer time for completion of the interceptive activity. There is no toxic effect at the particular dose level. Further work is in progress.

[1] The authors are thankful to Mr. P. P. GHOSH DASTIDAR, Medicinal Chemistry Division for the plant extracts. Thanks are also due to Indian Council of Medical Research and Council of Scientific and Industrial Research for granting scholarships to two of them (B.C. and A.D.) and to Prof. R. N. CHAKRAVARTI, Director of the Institute for encouragement.

[2] R. N. CHOPRA, S. L. NAYER and I. C. CHOPRA, *Glossary of Indian Medicinal Plants* (Council of Scientific and Industrial Research, New Delhi 1956), p. 24.

[3] K. R. KIRTIKAR and B. D. BASU, *Indian Medicinal Plants III* (L. M. Basu, Allahabad 1935), p. 2123.

[4] K. P. BISWAS and A. GHOSH, *Bharatiya Bonousadhi IV* (Calcutta University, India 1973), p. 985.

Studies in Relation to Endocrine Exophthalmos: The Biochemical Composition of Human Retrobulbar Connective Tissue

SANT P. SINGH and MILDRED NIKIFORUK[1]

Medical Research Service, Veterans Administration Hospital and Department of Medicine, Chicago Medical School, Downey (Illinois 60064, USA), 1 September 1975.

Summary. As part of studies on the pathogenesis of exophthalmos of Graves' disease, the biochemical composition of human retrobulbar tissue was investigated. The connective tissue was composed of 72.7% lipid, 23.8% water and 3.5% dried defatted tissue. Total tissue glycosaminoglycan (GAG) amounted to 0.18% of dried defatted tissue. Approximately 27% of the total hexosamine and 19% of the total tissue galactosamine were recovered in the GAG fraction. Cellulose microcolumn fractionation of GAG showed that the hyaluronic acid and dermatan sulfate were the two major GAG species.

Changes in retrobulbar connective tissue play a significant role in the production of exophthalmos of Graves' disease[2]. Major evidence of these observations is based upon studies in experimental animals. For example, the induction of exophthalmos in guinea-pig and fish was shown to result in increased metachromasia, hexosamine and hexuronic acid contents and ^{35}S-sulfate incorporation in the retrobulbar tissue[3]. Recently, we reported the use of the mouse orbital tissue as a method to study the etiology and pathogenesis of exophthalmos of Graves' disease[4]. To clarify the relevance of these observations to clinical exophthalmos, the present study was undertaken to characterize the biochemical composition of human retrobulbar connective tissue.

Materials and methods. 16 human cadavers ranging in age from 47 to 90 years (average 65 years) were studied. Within 48 h of death, retrobulbar tissue was obtained by cutting open a window in the orbital plate. Fibrofatty connective tissue was dissected free of the muscle fibres, cut into small pieces and weighed. Tissue segments were lyophilized, weighed, then passed through several changes of methanol-chloroform and dried to a constant weight in vacuo. The water and lipid contents were calculated from the wet, dried and dried defatted tissue weights. Individual eye samples were analyzed for water and lipid determinations and subsequently the tissues from both eyes were pooled for further studies.

Hydrolysis of the dried defatted tissue (approximately 10–12 mg) was performed with 4 *N* HCl, in a sealed glass tube, at 100 °C for 16 h in an oven. After dividing the hydrolyzed tissue into 2 aliquots, HCl from each aliquot was removed in vacuo over NaOH pellets. The residue was dissolved in 0.1 ml 0.1 *N* HCl for the determination of hexosamine[5] and galactosamine[6].

Glycosaminoglycans were liberated from the dried defatted tissue by proteolytic digestion with pronase[7] and recovered as potassium salt according to a previously described method[8]. Subsequently the crude glycosaminoglycans (GAG) preparation was dissolved in 0.075 *M* NaCl and divided into aliquots for chromatographic separation of GAG species and for the measurement of uronic acid[9], hexosamine[6] and galactosamine[5]. Each sample was desalted by dialysis against distilled water at 4 °C overnight prior to hexosamine and galactosamine determinations. GAG was fractioned on 0.8 × 16 cm cellulose columns according to the elution procedure of SVEJCAR and ROBERTSON[7]. In this method GAG are precipitated as cetylpyridinium chloride (CPC) complexes on cellulose

Table I. Water, lipid and protein content of retrobulbar connective tissue

	Water	Lipid	Dry weight	Protein
Mean ± SEM	23.8 ± 0.9	72.7 ± 1.0	3.5 ± 0.1	3.3 ± 0.2
Range	15.2 — 32.5	63.9 — 83.1	2.2 — 5.1	2.1 — 4.4

All values are expressed as percent of wet tissue weight. Retrobulbar connective tissue was removed within 4–48 h of death of 16 male humans and dissected free of extraorbital muscles before analysis.

[1] Acknowledgments. This study was supported by VA research funds. The authors are thankful to Dr. J. Ro and S. K. SINGH for obtaining the tissue samples, and to Mrs. RUTH M. BONOVICH for typing the manuscript.

[2] D. A. MCGILL and S. P. ASPER, New Engl. J. Med. *267*, 188 (1962).

[3] B. M. DOBYNS, in *The Pituitary Gland* (Butterworth, London 1966), p. 411.

[4] S. P. SINGH and J. M. MCKENZIE, Metabolism *20*, 422 (1971).

[5] C. CESSI and F. PILLEGO, Biochem. J. *77*, 508 (1960).

[6] C. CESSI and F. SERAFINI-CESSI, Biochem. J. *88*, 132 (1963).

[7] J. SVEJCAR and W. B. ROBERTSON, Analyt. Biochem. *18*, 333 (1967).

[8] S. P. SINGH and J. M. MCKENZIE, Endocrinology *85*, 952 (1969).

[9] T. BITTER and H. M. MUIR, Analyt. Biochem. *4*, 330 (1962).

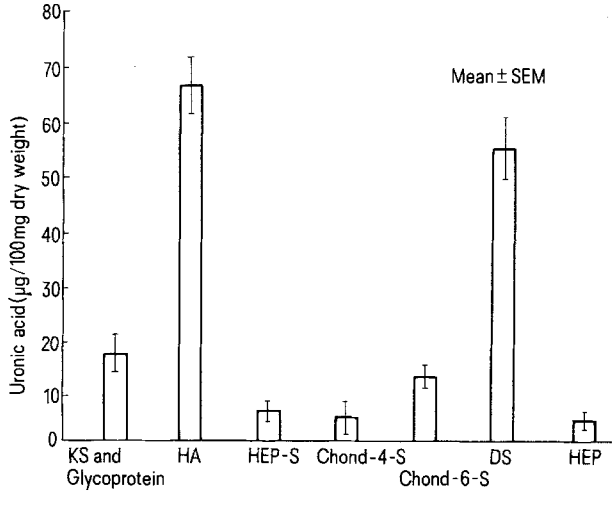
columns and sequentially eluted with increasing concentrations of salt, taking advantage of differential solubilities of GAG species in organic solvents and acid solutions. Individual eluates are considered to contain in sequence a) keratan sulfate and glycoprotein, b) hyaluronic acid, c) heparin sulfate, d) chondroitin-4-sulfate, e) chondroitin-6-sulfate, f) dermatan sulfate, and g) heparin. Although possible presence of other materials not named above in individual fractions could not be excluded, SVEJCAR and ROBERTSON[7] have confirmed the validity of these fractions in a variety of connective tissues. As a further check, GAG recovery from the columns was tested by applying pure and a mixture of known amounts of hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate and heparin (Sigma Chemical Co.). With the human orbital connective tissue, mean recovery of tissue GAG as uronic acid from the cellulose microcolumn was 83% ($n = 15$). Therefore, uronic acid value of each eluate was corrected accordingly.

Results. Table I shows the mean ± SEM values and the range of human retrobulbar connective tissue constituent, expressed as % wet tissue weight. Fat and water formed the bulk of the tissue, 72.7% and 23.8% (mean value), respectively. Dried defatted tissue constituted 3.5% of the whole tissue and most of it, i.e. 3.3%, was protein in nature. There was no correlation between concentration of fat and water in the retrobulbar tissue and the autopsy diagnoses. The analytical data for the dried defatted tissue and the isolated GAG are summarized in Table II. Approximately 27% of the total tissue hexosamine and 19% of the total tissue galactosamine were recovered in the GAG fraction. Glucosamine content calculated from the differences between hexosamine and galactosamine levels showed that 39% of the total tissue glucosamine originated from the GAG fraction. The ratio between galactosamine and glucosamine contents of the tissue GAG was 1:1.8.

Table II. Analysis of human retrobulbar connective tissue

	Whole tissue (dried, defatted)	GAG fraction	$\frac{\text{GAG}}{\text{Whole tissue}} \times 100$
Hexosamine	660 ± 20	180 ± 7	27 ± 1.0
Galactosamine	380 ± 35	71 ± 3	19 ± 3.0
Glucosamine	280 ± 50	110 ± 8	39 ± 4.0
Uronic acid	[a]	180 ± 8	[a]

Data presented are the mean ± SEM values of 13 experiments. Retrobulbar connective tissue from both orbits of individual humans was pooled for each experiment. All analyses were done as μg/100 mg dry defatted tissue. [a] Uronic acid estimation of whole tissue was not done due to the presence of proteins.



Glycosaminoglycan content of normal human retrobulbar connective tissue based on CPC-cellulose column separation (see text). Each bar represents Mean ± SEM value of 15 observations. KS, Keratan sulfate; HA, hyaluronic acid; Hep-S, heparin sulfate; Chond-4-S, chondroitin-4-sulfate; Chond-6-S, chondroitin-6-sulfate; DS, dermatan sulfate; Hep, heparin.

As is shown in the Figure, when the GAG harvested as potassium salt were further fractionated on a cellulose microcolumn, significant amounts of GAG were recovered in fractions 1, 2 and 6. Although contamination by other small molecular weight GAG could not be excluded, apparently fraction 1 material represented hyaluronic acid rather than glycoprotein or keratan sulfate. In a separate experiment, when commercially available hyaluronic acid (Sigma Chemical Co.) was applied to the cellulose microcolumn, 87% of it was eluted in fraction 2 (0.3 *M* NaCl) as expected but some was recovered in fraction 1. Therefore, the actual concentration of hyaluronic acid might be the sum of the material eluted in fractions 1 and 2.

Next to hyaluronic acid, dermatan sulfate was found to be the major GAG. Other chromatographic fractions contained GAG in relatively small amounts. In terms of percentage of the total uronic acid eluted from the columns, the relative distribution of the individual GAG was as follows: hyaluronic acid 50.8% (sum of fractions 1 and 2), dermatan sulfate 31.1%, chondroitin-6-sulfate 8.0%, chondroitin-4-sulfate 3.4%, heparin sulfate 3.9% and heparin 2.6%.

Discussion. The results of the present study show that water and fat are the major constituents of the human retrobulbar connective tissue. The dried defatted tissue has relatively low hexosamine content, of which approximately 27% is derived from the tissue glycosaminoglycans (GAG).

The microfractionation procedure used in the present investigations separate different fractions of GAG with a reasonably good reproducibility, but does not permit any definite conclusion to be drawn concerning the chemical nature of each fraction. Nevertheless, we have confirmed the findings of PRAME, GARDELL and ANTONOPOULOS[10] that hyaluronic acid and dermatan sulfate are the major GAG fractions. The identity of the fraction 1 eluted with 1% CPC has not been finally established. Apparently fraction 1 had gross contamination with hyaluronic acid or GAG of low molecular weight.

Our data suggest that relatively small amounts of chrondroitin-4-sulfate, chondroitin-6-sulfate, heparin sulfate and heparin are present in the human retrobulbar connective tissue. PRAME et al.[10] reported that substantial amounts of GAG were present in fraction 3 eluted with 0.28% $MgCl_2$. This solvent has been shown to elute heparin sulfate. In our study only 4% of total uronic acid was recovered in fraction 3. The reasons for this discrepancy are unclear, although postmortem changes in the molecular weight of the GAG might partly be a responsible factor.

[10] G. PRAME, S. GARDELL and C. A. ANTONOPOULOS, Expl Eye Res. *5*, 79 (1966).