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# URINARY HEPARAN SULPHATE IS INCREASED IN NORMOALBUMINURIC DIABETIC PATIENTS

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**Abstract** Forty-nine normoalbuminuric diabetic patients were studied: 22 males and 27 females, in whom urinary heparan sulphate (HS), albuminuria, creatininemia, creatininuria, creatinine clearance, HbA1c and arterial pressure (AP) were determined. Two groups were discerned: group 1, Type 1 DM, diabetic cases (n = 16); and group 2, Type 2 DM diabetic cases (n = 33). Patients were compared with 24 healthy controls: 12 men and 12 women, who showed a mean value ± SD of 0.36 ± 0.18 mg/24 h HS with significant differences between males and females (0.43 ± 0.15 versus 0.28 ± 0.17, respectively; p = 0.02). The total population of diabetic cases rendered a mean of 0.68 ± 0.44 and comparison with controls proved highly significant (p < 0.001). Globally, male patients had a mean of 0.82 ± 0.48 and females 0.54 ± 0.35, with p < 0.02. Group 1 and 2 values of HS were not significantly different. HS levels failed to correlate either with age, body mass index (BMI), time since onset of diabetes, albuminuria, creatininemia, creatininuria, creatinine clearance, HbA1c or arterial hypertension. To conclude: both normal and diabetic males eliminate a greater quantity of HS than females. Normoalbuminuric diabetic patients of both types eliminate a greater quantity of HS regardless of arterial pressure and time since onset of diabetes.

**Resumen** ***Aumento de heparan sulfato urinario en diabéticos normoalbuminúricos.*** Se estudiaron 49 pacientes diabéticos: 22 hombres y 27 mujeres. Se determinaron: heparán sulfato (HS) urinario, albuminuria, creatininemia, creatininuria, clearance de creatinina, $HbA_{1c}$ y presión arterial (PA). Los pacientes se clasificaron en dos grupos: grupo 1, portadores de Diabetes Mellitus (DM) Tipo 1 (n = 16), y grupo 2, portadores de DM Tipo 2 (n = 33). Se utilizaron 24 sujetos sanos como controles, 12 hombres y 12 mujeres los cuales mostraron un valor medio ± DS de HS de 0.36 ± 0.18 mg/24 hs. Se hallaron diferencias significativas entre hombres y mujeres; 0.43 ± 0.15 versus 0.28 ± 0.17 respectivamente; ( p = 0.02). La población diabética dio un valor medio de 0.68 ± 0.44 el cual comparado con el de los controles mostró una significancia de p < 0.001. Los pacientes hombres tuvieron un valor medio de 0.82 ± 0.48 y las pacientes mujeres 0.54 ± 0.35; p < 0.02. No se encontraron datos significativamente diferentes entre los grupos 1 y 2. Los valores de HS no se correlacionaron con la edad, índice de masa corporal (IMC), tiempo transcurrido desde el comienzo de la diabetes, albuminuria, creatininemia, creatininuria, clearance de creatinina, $HbA_{1c}$ ni PA. Se concluye que: tanto en controles como diabéticos los hombres eliminan más HS urinario que las mujeres y que los pacientes diabéticos normoalbuminúricos eliminan más HS urinario que los controles, independientemente del tiempo transcurrido desde el inicio de la diabetes y la PA.

**Key words:** heparan sulphate, diabetes, normoalbuminuria

Diabetic nephropathy is the first cause of admission to supportive renal treatment, and is represented by 30-40% of insulin-dependent (Type 1)[1] and 5-10% of non-insulin-dependent (Type 2) patients in the course of diabetes[2].

In developed countries 30-40% of patients on hemodialysis are diabetic, which carries an extremely high social and economic burden[3, 4]. Over the last few years, attempts have been made to detect early markers of nephropathy in order to initiate prompt treatment and thus avoid the development of renal insufficiency, since patients with impaired renal function present greater morbidity and mortality than the general population[5].

In spite of the efforts and multiple studies, to date it has not been possible to identify reliably the factors that allow prevention of this pathology. Microalbuminuria is considered an early marker of renal lesion, and its determination is useful to decide the initiation of treatment for incipient nephropathy[6]. In order to optimise such therapy it has been attempted to detect diverse even earlier markers than microalbuminuria, such as prorenin[7] and Na/Li cotransport[8].

The glomerular barrier is made up of fenestrated endothelium, basal membrane and endothelial and epithelial cells. In turn, the glomerular basal membrane

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(GBM) consists of collagen IV and V, laminin, enactin, heparan and chondroitin sulphate proteoglycans[9]. This barrier is selectively permeable to the passage of molecules (albumin, globulins), restricting the passage according to their load, size and charge. Membrane heparan sulphate (HS) seems to play a major role in such restriction, as confirmed by injecting monoclonal antibodies against the glycosaminoglycan (GAG) chain of HS[10, 11], with alteration of GMB contents and appearance of albuminuria[12, 13].

In other studies, alterations have been observed in the selective permeability of the membrane through urine analysis, where a modification in the immunoglobulin/albumin ratio was found, as well as differences between neutral immunoglobulins and those charged negatively[14].

HS is synthesised by several cell types: vascular endothelium, smooth muscle and glomerular or mesangial epithelial cells. Its function is to maintain the structural integrity of the GMB and its negative charge. It binds to lipoproteinlipase and superoxide dismutase, which produces a regulatory effect on lipids and thrombogenesis, besides affecting the regulation of cellular growth[15].

The hypothesis advanced at Steno suggests that diabetes produces a modification in HS metabolism and that such alteration is the key to renal and vascular pathology[16].

Several experiments carried out in diabetic rats and humans show that urinary excretion of GAGs and more specifically of HS are increased. Reddi et al[17] measured HS in diabetic rat glomeruli and observed a decrease in level.

In Type 1 DM patients with microalbuminuria exceeding 200 mg/24 hours, Vernier et al[40] detected a 30-40% decrease in anionic sites digestible by heparinase in the kidney.

Bonavita et al.[22] studied HS urinary excretion in 15 diabetic juvenile, 15 diabetic adults and 44 normal controls.It was found that youngsters of both sexes excrete more HS than adults and that both normal males and diabetic juveniles eliminate more HS than females. Diabetic adults of both sexes also eliminate a greater percentage of HS, more than normal controls.

Baggio et al.[23] determined urinary GAGs in 40 Type 1 patients with normoalbuminuria and found that they excreted a greater quantity of GAGs.

Perez Blanco et al.[24] investigated urinary GAG excretion in healthy individuals, patients with neither hypertension nor microalbuminuria, those without hypertension but with microalbuminuria and those with both microalbuminuria and hypertension. The latter cases showed the highest values; in the second group GAGs were greater than in controls and in the third levels were significantly higher.

In the present work we attempted to determine urinary HS elimination in normoalbuminuric Type 1 DM and Type 2 DM patients (albuminuria < 30 mg/24 hs), and its correlation with risk factors of diabetic nephropathy, time since onset of diabetes, glycosylated hemoglobin (HbA1c) and arterial hypertension.

## Patients and Methods

Forty-nine diabetic patients, all normoalbuminuric (albuminuria < 30 mg/24 hs), were studied to determine HS in urine. There were 27 females and 22 males. Two groups were discerned according to the American Diabetes Association[25]: Group I (Type 1 DM n = 16); and Group 2 (Type 2 DM n = 33). Patients were compared with 24 healthy controls, comprising 12 males and 12 females.

Diabetic patients were free of any associated pathology, liver or thyroid disease, decompensated cardiac insufficiency or infectious process. Pregnant women were excluded.

Clinical features of controls and diabetic patients are shown in Table 1. Twenty four hour urine was collected without addition of preserving agents and cooled to 4°C. Sample collection was contraindicated in patients who were febrile, while relative impediments included physical exercise and liquid intake during the night.

Briefly, HS was determined as follows[22]: 40 ml aliquots were precipitated with bromo-hexadecyltrimethylammonium (cetrimide USP, Sigma). The precipitate was removed by repeated ethanol washes. Material insoluble in ethanol was dissolved in 2.5 ml of water, centrifuged and passed through a 1 x 4 cm 50 x 2, $H^+$ Dowex column, and thereafter eluted with distilled water. The acidic effluent was neutralised with 0.5 N NaOH and lyophilised; then an aliquot of the lyophilised material reconstituted with distilled water was taken and an equal volume of 1 N HCl was added, heating the mixture at 110°C during 2 h; duplicate aliquots were then drawn to measure glucosamine. For this reaction, desulphation and deamination with nitrous acid was performed according to Lagunoff and Warren[26]; lastly, produced 2-5 anhydrohexose was quantified with chloro-3-methyl-2-benzothiazolone according to Smith and Gilkerson[27], using a glucosamine standard. Results are expressed in mg of glucosamine per 24 hours. Method CV% was 19.4%.

Albuminuria was determined in 24 hours urine by radioimmunoassay using a commercial device (Albumin RIA, DPC, USA)[28]. In this technique the patient's urinary albumin competes with $^{125}$I-labeled urine for the binding sites of an antibody. After incubation free and bound fractions were

TABLE 1.– *Clinical features and laboratory findings in control subjects and diabetic patients*

| Feature | Controls (n=24) | Diabetic patients (n=49) |
|---|---|---|
| Sex ratio (F/M) | 12/12 | 27/22 |
| Age in years[a] | 40.61 ± 17.09 | 53.44 ± 19.02 |
| Range in years | 18-73 | 17-83 |
| BMI kg/m$^2$ [b] | 25.2 ± 3.1 | 27.1 ± 4.6 |
| Albuminuria mg/day[c] | 5.54 ± 2.87 | 12.0 ± 11.8 |
| HbA1c%[d] | 6.5 ± 2.8 | 7.3 ± 2.3 |
| Blood creatinine mg/dl[e] | 0.87 ± 0.21 | 0.96 ± 0.19 |
| Creatinine clearance %[f] | 92.12 ± 27.0 | 82.97 ± 30.24 |

*Laboratory data are expressed in mean values ± SD and SEM.*
[a] *p < 0.008;* [b] *NS;* [c] *p < 0.001;* [d] *NS;* [e] *p < 0.025;* [f] *NS*

separated with an anti gamma globulin diluted with polyethylenglycol. The bound fraction was precipitated by centrifugation and quantified in a gamma counter. Urine sample concentrations were determined in comparison with a calibration curve (from 0 to 60 µg/ml) that was processed simultaneously. Method detection limit is 0.3 µg/ml. Cut-off value of normoalbuminuria was taken as 30 mg/24 hs. Method CV% was 2.8%.

Besides, creatinine in blood was determined by a reaction with picric acid in alkaline medium to render a coloured complex which is quantified by spectrophotometric reading[29]. Method CV% was 2.4%. For determination in urine, samples were diluted 1/10 and 1/50 in distilled water.

HbA1c was determined with a DCA 2000 commercial kit from Bayer Diagnostics, which used a system based on the inhibition of the agglutination of latex particles coated with a specific monoclonal antibody against HbA1c. Reference values were 4.3-5.7% of total hemoglobin. Method CV% was 3.3.

Arterial pressure was taken with a sphingomanometer after 5 minuts resting and defined as greater than 140 mm Hg and greater than 90 mm Hg as arterial hypertension.

Statistical analysis. Quantitative variables were expressed as means ± SD. SD values between group pairs were analysed by Student's t test for independent samples or by the non-parametric Kruskal-Wallis method when irregular distribution were present. The correlation between quantitative variables was studied by Pearson's linear regression method.

A multiple linear regression with dependent variable HS was carried out with the following independent variables: age, gender, creatinine, arterial hypertension and time since onset of diabetes, as well as 24 h microalbuminuria and albuminuria.

## Results

Table 1 provides clinical and laboratory data for diabetic and control groups. Figure 1 shows HS data distribution observed in diabetic patients and controls. Mean HS value ± SD for controls was 0.36 ± 0.18 mg/24 hs and 0.68 ± 0.44 mg/24 hs for the total diabetic population.

Mean HS value in the control group displayed a significant difference according to the t test between males and females (0.43 ± 0.15 versus 0.28 ± 0.17 mg/24 hs respectively; p = 0.02) (Fig. 2). In the diabetic group, males had a mean of 0.82 ± 0.48 mg/24 hs and females 0.54 ± 0.35 mg/24 hs (p < 0.02).

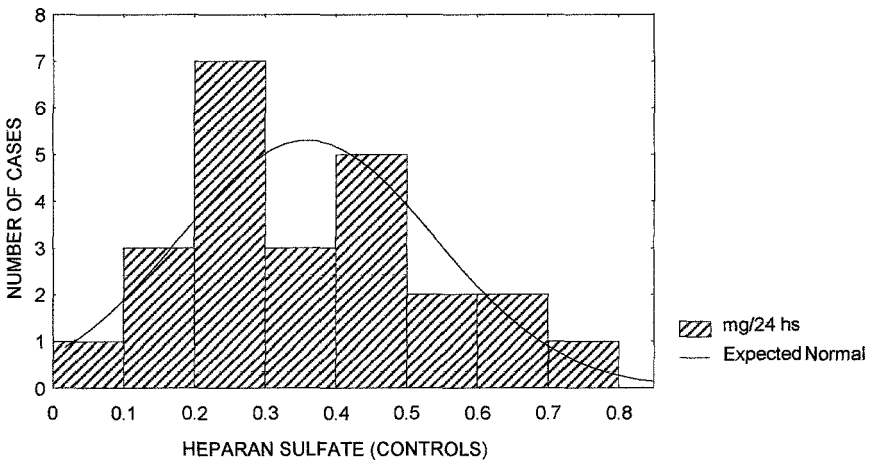


Fig. 1.– a. Heparan sulphate distribution in 24 control subjects.

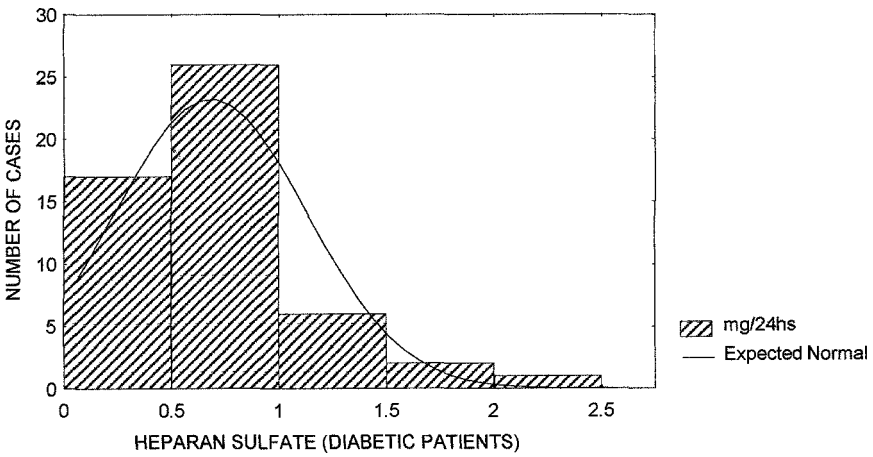


Fig. 1.– b. Heparan sulphate distribution in 49 diabetic patients.

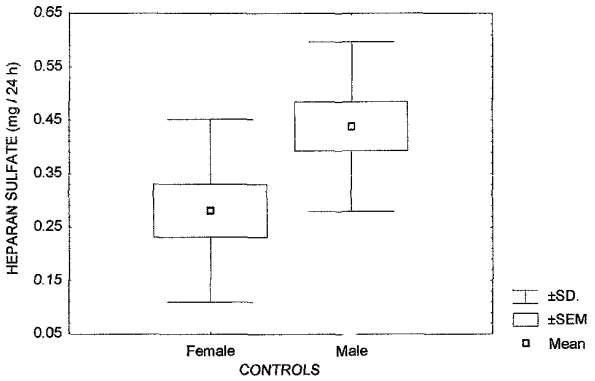


Fig. 2.– a. Urinary excretion of heparan sulphate (HS) in female (F) and in male control subjects (M). Data are expressed as mean values ± SD and SEM in mg/24 hours of urinary HS, with p = 0.02.

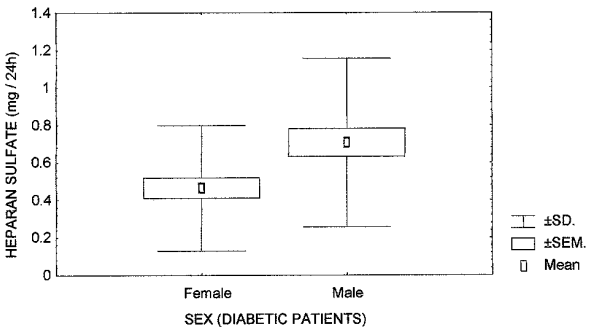


Fig. 2.– b. Urinary excretion of heparan sulphate (HS) in diabetic females (F) and in males (M). Data are expressed as mean values ± SD and SEM in mg/24 hours of urinary HS, with p < 0.02.

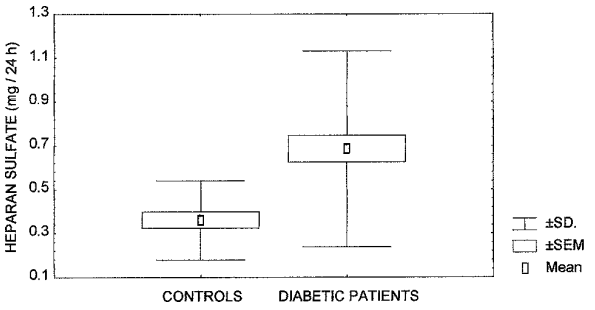


Fig. 3.– Urinary excretion of heparan sulphate (HS) in healthy controls (C) and in normoalbuminuric diabetic patients (D). Data are expressed as mean values ± SD and SEM in mg/ 24 hours of urinary HS, with p = 0.001.

If HS levels are compared in diabetic men versus male controls, the significant difference in greater elimination in the former is maintained (p = 0.001), as well as in diabetic women versus their controls (p = 0.02), both according to Kruskal-Wallis (data not shown).

Figure 3 shows mean HS values in controls versus diabetic patients with a highly significant p < 0.001

When adjustment was made for age, diabetic patients presented a mean value of 0.66 mg/24 hs and controls 0.35 mg/24 hs; with p = 0.002 (data not shown).

On comparing HS value in the Type 1 DM versus the Type 2 DM diabetic group (0.75 ± 0.52 mg/24 hs and 0.60 ± 0.36 mg/24 hs, respectively), the difference was not significant.

There was no correlation between HS level with factors such as age, time since onset of diabetes, BMI, albumin level and HTA, or with creatinine clearance, while a positive correlation with HbA1C was not found.

## Discussion

According to Mogensen[6] incipient nephropathy has its onset roughly towards the fifth year following the development of microalbuminuria in the course of diabetes. The features of its course are predictable, with microalbuminuria progressing to proteinuria, then to azotemia and finally to chronic renal insufficiency (CRI), with speeding up of successive stages when the management of arterial pressure or metabolic control is unsuitable.

Over the last few years, research has been focused on studying early markers of chronic complications in order to determine accurately which patients are prone to develop such disorders. From the clinical point of view, microalbuminuria[30] has been the component taken into account as a marker of renal lesion[31], of nephropathy development[32], or cardiovascular risk and of generalized vascular lesion[33].

The Steno hypothesis[16], as well as work demonstrating the greater prevalence of renal complications in familial groups[34], in addition to metabolic and haemodynamic factors, support the existence of genetic factors in its development.

Heparan sulphate proteoglycan (HS-PG) is a major component of the glomerular basal membrane (GBM) that plays a leading role as an organizing and structural molecule[35]. The presence of this strongly negative molecule is essential to maintain the selective permeability of GMB[36]. The loss of HS has been associated with proteinuria in numerous glomerulopathies[37, 38]. Despite their normoalbuminuric status, kidney biopsy in patients with diabetes during this period discloses alterations in the extracellular matrix with GMB thickening and an increase in the mesangial matrix. Changes in the extracellular matrix with loss of the heparan sulphate proteoglycan seem to play a leading role in proteinuria[35].

Rohrabach et al reported reduction in HS synthesis in diabetic rats[36], while others demonstrated a decrease in tissue by means of antibodies directed against the HS-PG in the basal membrane[10].

The results obtained in our study in urine from normoalbuminuric diabetic patients show increased levels of HS, significantly greater in men that in women, and such difference persists with age adjustment. It may therefore be concluded that age difference seems not to be a factor influencing the increase in urinary HS.

Other studies evaluating the elimination of diverse proteoglycan components in normoalbuminuric patients also displayed some differences. Craddock et al[20] studied in urine the fraction designated Astrup, consisting mainly of acid mucopolysaccharides with a predominance of hexuronic acid and hexosamine. They also found that such fraction is excreted in greater proportion by diabetic subjects with normal renal function that by normal controls.

Lubec et al[21] evaluated urinary GAGs by means of the reaction with carbazol in diabetic youths, to find that they excreted more GAGs as compared with controls. They also determined HbA1c and recorded a highly significant correlation with GAGs ($r = 0.7$; $p < 0.01$).

Reddi[17] studied Wistar rats, to find that the group treated with streptozotocine presented lower glomerular GAG and HS concentrations as evidenced by reduced incorporation of $^{35}S$ sulphate, which would indicate decreased glomerular synthesis. Furthermore, these rats presented greater urinary HS elimination than controls. The correlation of urinary HS versus proteinuria was significantly positive. The author concluded that glomerular HS synthesis was reduced concomitantly with increased urinary elimination of the proteoglycan.

The relationship between HS excretion and albuminuria remain unclear. McAuliffe et al.[18] measured urinary HS excretion in diabetic and non diabetic subjects with varying degree of albuminuria. The urine was collected with the subject at rest over a 2.5 h period after a 300 ml water load. Categorizing for albuminuric status shows that the diabetic micro and macroalbuminuric groups have a significantly higher HS excretion rate than non diabetic subjects.

In Shield et al[19] article both groups of diabetic patients with and without microalbuminuria (15 µg/min in at least two of three consecutive urine collections) had significantly elevated excretion HS when compared to normal individual, there was no difference in HS excretion between diabetic subjects with and without microalbuminuria.

It has been debated whether the mechanism of HS decrease is due to an alteration in synthesis, to a change in its composition or to the loss of the membrane proteoglycan by urine; however, in every case there is a decrease in HS concentration at the glomerular barrier.

The correlation in our study between albuminuria and HS excretion was not significant. Interestingly, though, the albuminuria level (significantly different in diabetic patients vs controls, with $p < 0.001$), while remaining within ranges considered normal, should be regarded as an influential factor in the elevation of urinary HS elimination.

Converting enzyme inhibitors have been widely used for their multiple effects in delaying the progression of renal function decline. Reddi et al[35] observed that these drugs increased GBM HS and reduced its elimination, a mechanism perhaps operative on the GBM which favours its effect on microalbuminuria, also supporting the major role played by HS in the development of diabetic nephropathy.

Heparan sulphate is a strong inhibitor of mesangial growth and its reduction has been demonstrated in diabetic patients with mesangial expansion and clinical nephropathy[39]. In other work in humans a negative correlation was demonstrated between GBM anionic groups and albumin excretion[40].

No correlation was found in our study with risk factors such as arterial pressure levels. The high pressure seems related with the deterioration in renal function but its increase is described when urinary albumin exceeds 100 mg/24 hs[40]. Our patients had less albuminuria.

In other studies performed in normoalbuminuric patients high arterial pressure levels were only found when evaluation was carried out by more sensitive methods such as pressurometry[41], not employed here.

In our population, there was no correlation between HS and the time since onset of diabetes and no positive correlation with HbA1c was found. In all likelihood, other as yet undetermined variables, particularly genetic factors, influence urinary HS elimination. On the other hand, there was a trend that failed to reach statistical significance with creatinine values.

The leading role of the glycosaminoglycan in the pathogenesis of the nephropathy would gain support from work showing the possibility of reducing microalbuminuria by means of oral therapy with glycosaminoglycan[42].

The increase in urinary HS in our study failed to correlate with any of the risk parameters considered for the development of the diabetic nephropathy such as time since onset of diabetes, arterial hypertension or HbA1c. This finding agrees with the theory of Steno suggesting that extracellular matrix impairment alters endothelial GBM in a subgroup of diabetic patients with greater genetic susceptibility to develop the renal complication[43]. The answer could be obtained through a study with a greater number of patients followed up prospectively, confirming the subsequent appearance of microalbu-minuria in those presenting increased HS.

The loss of HS-PG modifies capillary permeability leading to an increase in albuminuria levels. Perhaps the early appearance of HS in urine could predict patients likely to present microalbuminuria, but this would have to be studied prospectively during follow-up of patients showing increased HS levels.

In conclusion, our results show that both normal and diabetic males eliminate a greater quantity of HS than females, normoalbuminuric diabetic patients of both types eliminate a greater quantity of HS without any correlation with the evaluated risk factors.

The increased HS in longitudinal follow-up of this subgroup and confirmation of their progress towards microalbuminuria would support the leading role of glycosaminoglycan in the pathogenesis of diabetic nephropathy.

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*[...] Most people really waste the time of enforced idleness which is such an inevitable accompaniment of war. It is greatly to the credit of the contributors here that they have fought mental lethargy to the point of creating this valuable composite statements upon the cell. The spirit is indeed hard to crush: the weakness and strength of the flesh rest upon organized chemistry.*

[...] La mayoría de las personas realmente desperdicia el tiempo de forzada inactividad que es inevitable acompañamiento de la guerra. Es grande el mérito de los que contribuyeron aquí que han combatido el letargo mental hasta el punto de crear esta valiosa combinación de exposiciones sobre la célula. El espíritu es verdaderamente duro de aplastar; la debilidad y la fuerza de la carne se apoyan sobre la química organizada.

R. A Peters

Foreword (Prefacio) de *Cytology and Cell Physiology*, edited by Geoffrey Bourne.
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