# THE QUANTITATION OF GLUCOSAMINE AND GALACTOSAMINE IN GLYCOPROTEINS AFTER HYDROLYSIS IN *p*-TOLUENESULPHONIC ACID

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## 1. Introduction

The amino acid composition of glycoproteins can be reliably estimated, after acid hydrolysis, by the use of an amino acid analyser and the neutral sugar composition, after methanolysis and trimethylsilylation, by gas-liquid chromatography (g.l.c.). The estimation of hexosamines is less satisfactory, the main problem being the quantitative release of these compounds from glycoproteins without significant destruction. Hydrolysis in HCl is often used, but as pointed out by Marshall and Neuberger [1], if a glycoprotein is hydrolysed under conditions which are sufficient to release the hexosamines without causing their destruction (e.g. 4 M HCl at 100°C for 4 h), the hydrolysate will contain peptides and therefore is not likely to be suitable for direct application to the column of an amino acid analyser. If on the other hand the glycoprotein is hydrolysed under conditions which are rigorous enough to cleave all the peptide bonds, there is considerable destruction of the hexosamines (about 50% loss in 6 M HCl at 110°C in 24 h). An alternative is to use g.l.c. after methanolysis and trimethylsilylation [2] but this procedure is not always as reliable for amino sugars [3] as it is for neutral sugars, which means that an alternative method for the analysis of hexosamines should be of value even in those laboratories that have access to g.l.c.

Liu and Chang [4,5] recommended the replacement of 6 M HCl by 3 M *p*-toluenesulphonic acid for the hydrolysis of proteins and glycoproteins at 110°C, as this reagent caused hardly any destruction of tryptophan while releasing amino acids at the same rate as HCl. These authors also reported in a footnote recoveries of glucosamine of between 70% and 80% in hydrolysates of glycoproteins. We have further investigated the possibilities of this method and have found that when glycoproteins are hydrolysed in 3 M *p*-toluenesulphonic acid for 24 h at 100°C rather than at 110°C there is an almost quantitative recovery of glucosamine and galactosamine which can be subsequently estimated on an amino acid analyser. Having found this method to be satisfactory for the analysis of various glycoproteins [6,7] we felt it necessary to give full details of the procedure and proof of its reliability.

## 2. Materials and methods

*p*-Toluenesulphonic acid (microanalytical grade) was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, UK. Sugars used as standards were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., UK. Human [8] and rabbit [9] Tamm-Horsfall urinary glycoproteins were prepared by Dr Anne M. S. Grant, Department of Chemical Pathology, St Mary's Hospital Medical School, London W2, UK. Soybean agglutinin was prepared by affinity chromatography [10]. Hen ovomucoid was obtained from Worthington Biochemical Corp., Freehold, N. J., USA and hen lysozyme, hen ovalbumin, bovine fetuin and human serum transferrin from Sigma Chemical Corp., Norbiton Station Yard, Surrey, UK. Glycoproteins from commercial sources were used without further purification. Other chemicals were of analytical grade or the highest purity available.

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2.1. *Elson–Morgan reaction*

This was carried out according to the modified method of Rondle and Morgan [11] after hydrolysis of the glycoprotein for 4 h at 100°C in 4 M HCl in vacuo followed by removal of the HCl over NaOH in vacuo. It was necessary to introduce a correction factor for the chromogen produced during hydrolysis by the reaction of neutral sugars with some amino acid residues (particularly lysine) of the protein [12]. This was obtained from a correction curve produced by hydrolysing hen egg-white lysozyme in the presence of varying amounts of mannose and subjecting the hydrolysates to the Elson–Morgan reaction. The colour produced by the hydrolysates of the standard glycoproteins in the Elson–Morgan reaction was then corrected according to the percentage of neutral sugar (known from g.l.c.) in these glycoproteins.

2.2. *Gas-liquid chromatography*

The method of Chambers and Clamp [13] was used. This involved methanolysis of a dry sample of the glycoprotein with mannitol as an internal standard in 1 M methanolic-HCl for 4 h at 90°C followed by re-acetylation and trimethylsilylation. The Tamm–Horsfall glycoproteins, which contain traces of lipid were extracted with hexane after methanolysis. A Perkin–Elmer F.11 gas chromatograph fitted with dual columns and flame ionisation detection was used for the analyses.

2.3. *Preparation of acid hydrolysates*

Glycoproteins were dried in vacuo in hydrolysis tubes with *p*-fluorophenylalanine as an internal standard. The hydrolysing acid (0.5 ml), either 6 M HCl or 3 M *p*-toluenesulphonic acid was added, and the solution was thoroughly de-aerated by repeated freezing and thawing under vacuum. The tubes were then sealed and heated in an oven at either 100°C or 110°C for 24 h to 72 h according to the conditions of the experiment. HCl was removed from the hydrolysate over NaOH in vacuo, *p*-toluenesulphonic acid was partially neutralised by the addition of 1 ml of 1 N NaOH as recommended by Liu [4,5] for direct application to the analyser.

2.4. *Determinations with the amino acid analyser*

Analyses were done on a Locarte amino acid analyser fitted with an automatic loading attachment. For the quantitative estimation of glucosamine and galactosamine we recommend elution of the hydrolysate from the 23 cm column of the analyser with the standard pH 4.25 (0.2 M $Na^+$) buffer of Moore and Stein [14] at 50°C. This fully resolves (in order) methione, isoleucine, leucine, tyrosine, phenylalanine, *p*-fluorophenylalanine, glucosamine and galactosamine from each other and from tryptophan and its breakdown products which frequently interfere with the analysis of hexosamines in other systems such as that of Liu [4,5]. For the complete amino acid analyses the system of Mayes et al. [15] can be used as it allows the separation of glucosamine and galactosamine from the amino acids, but it is only suitable for the quantitative estimation of the hexosamines in glycoproteins with a low tryptophan to hexosamine ratio or for simple glycopeptides.

## 3. Results

3.1. *Survival of added glucosamine and galactosamine during hydrolysis of lysozyme*

A stock solution of 1 mM glucosamine–HCl, 1 mM galactosamine–HCl and 1 mM *p*-fluorophenylalanine was prepared and a portion of this was added to an equal volume of a solution of lysozyme (1.0 mg/ml) to make a second stock solution. Separate portions (100 μl) of this second stock solution were heated in 3 M *p*-toluenesulphonic acid for 24, 48 or 72 h at both 100°C and 110°C in duplicate. The hydrolysates were then analysed for their amino sugar content in comparison with the original first stock (table 1). This

Table 1

The survival of added hexosamines during hydrolysis of lysozymes in 3 M *p*-toluenesulphonic acid at 100°C and 110°C

| Temperature of hydrolysis | Sugar | Percentage of hexosamine remaining | | |
|---|---|---|---|---|
| | | Time of hydrolysis | | |
| | | 24 h | 48 h | 72 h |
| 100°C | GlcN | 95.6 | 91.3 | 85.2 |
| 100°C | GalN | 94.3 | 85.0 | 79.3 |
| 110°C | GlcN | 82.1 | 70.0 | 48.8 |
| 110°C | GalN | 77.8 | 58.4 | 33.7 |

For conditons see Methods.

Table 2
A comparison of hydrolysis in *p*-toluenesulphonic acid (pTSA) with other methods for the estimation of the hexosamine content of glycoproteins

| Glycoprotein | Sugar | Method of analysis | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Elson–Morgan after 4 M–HCl at 100°C | Methanolysis and g.l.c. | Amino acid analyser 6 M HCl 110°C | Amino acid analyser 3 M pTSA 100°C | Literature values | Molecular weight (subunit) | References |
| Rabbit Tamm- | GlcN | 53 | 53.7 | 21.7 | 57.5 | 56.9 | 84 000 | [3,9] |
| Horsfall | GalN | | 2.3 | 0.2 | 0.6 | 0.9 | | |
| Human Tamm- | GlcN | 27 | 34.5 | 12.9 | 38.6 | 34.4 | 79 000 | [3,8] |
| Horsfall | GalN | | 5.6 | 2.6 | 4.1 | 5.6 | | |
| Hen Ovomucoid | GlcN | 31 | 28.2 | 9.5 | 25.6 | 24 | 27 000 | [16] |
| | GalN | | 0.0 | 0.0 | 0.0 | 0.0 | | |
| Bovine Fetuin | GlcN | 15 | 12.9 | 7.1 | 13.9 | 15.7 | 48 000 | [17] |
| | GalN | | 2.7 | 1.6 | 2.4 | 3.1 | | |
| Human serum | GlcN | 8.9 | 7.5 | 4.2 | 9.2 | 8 | 83 000 | [18] |
| Transferrin | GalN | | 0.0 | 0.0 | 0.0 | 0.0 | | |
| Soybean | GlcN | 3.4 | 1.9 | 1.6 | 3.1 | 3 | 30 000 | [19] |
| Agglutinin | GalN | | 0.0 | 0.0 | 0.0 | 0.0 | | |
| Hen Ovalbumin | GlcN | 7.6 | 5.1 | 3.0 | 6.2 | 5 | 46 000 | [20] |
| | GalN | | 0.0 | 0.0 | 0.0 | 0.0 | | |

For details of the procedure see the Methods section. All figures are expressed as residues per (subunit) molecular weight.

showed that the rate of destruction of the amino sugars was about 20% per 24 h at 110°C but only about 5% per 24 h at 100°C. In preliminary experiments it was found that the addition of tryptamine (which is added to protect tryptophan in the procedure of Liu [4,5]) to the 3 M *p*-toluenesulphonic acid did not improve the recovery of hexosamines so it was not normally included in the hydrolysates.

### 3.2. *Comparison of various methods for the estimation of hexosamines*

Following the indication that hydrolysis of glycoproteins in *p*-toluenesulphonic acid at 100°C would be a suitable method for the accurate estimation of their hexosamine content we analysed seven glycoproteins both by this method and by three other methods for comparison (table 2). The other methods were (i) hydrolysis in 6 M HCl for 24 h at 110°C followed by analysis on the amino acid analyser, (ii) hydrolysis in 4 M HCl for 4 h at 100°C followed by the Elson–Morgan reaction and (iii) methanolysis followed by trimethylsilylation for g.l.c. Details of these procedures are given in the Methods section. Duplicate aliquots of the seven glycoproteins were taken for analysis by the four methods with appropriate internal standards. It can be seen from table 2 that the analyses of the *p*-toluenesulphonic acid hydrolysates are mostly equal to or higher than the estimations by g.l.c. and the Elson–Morgan method. The figures from the literature, which are the highest reliable figures, are in most cases exceeded by the analyses of the *p*-toluenesulphonic acid hydrolysates.

### 3.3. *Recommended procedure for the analysis of hexosamines in glycoproteins*

The recommended procedure is to take aliquots from a solution of the glycoprotein, add *p*-fluorophenylalanine as an internal standard for samples intended for the amino acid analyser, and mannitol for any samples intended for g.l.c. and dry them over $P_2O_5$ in vacuo. The sample intended for hexosamine analysis is then dissolved in 0.5 ml 3 M *p*-toluenesulphonic acid, de-gassed and sealed in vacuo (see Methods) and hydrolysed for 24 h at 100°C. 1 M NaOH (1 ml) and 0.5 ml water are added to the hydrolysate and samples of this solution are applied to the amino acid analyser and eluted with the pH 4.25 buffer system (see Methods). The results for the hexosamine analyses can then be related by means of the *p*-fluorophenylalanine internal standards to analyses of samples hydrolysed at 110°C which are used to assess the total amino acid content. We found that hydrolysis at 100°C fully released phenylalanine from the glycoproteins that we examined, and therefore the 100°C and 110°C hydrolysates could also be related through this amino acid.

## 4. Discussion

The procedure recommended above has the advantages over other systems commonly used for hexosamine analyses that it is quantitiative, unambiguous for both glucosamine and galactosamine, and that the analyses are easily related to those of the amino acids and the neutral sugars. In comparison, hydrolysis in 6 M HCl for 24 h at 110°C is inaccurate as extrapolations from a rate of destruction of 50% in 24 h are required. Hydrolysis in 4 M HCl for 4 h at 100°C followed by an Elson–Morgan estimation has the disadvantage that correction for the reaction of neutral sugars with amino acids during hydrolysis is necessary and that it is not possible to discriminate between glucosamine and galactosamine. The procedure of methanolysis and trimethylsilylation of sugars for g.l.c. [2] is generally satisfactory for neutral sugars but for the estimation of the hexosamines the conditions seem to be more critical [3]. Particular difficulties have been found in this laboratory in estimating the hexosamine content of lymphocyte plasma membranes by this method [7]. In addition it is often difficult to resolve the peaks due to galactosamine from the multiple peaks of glucosamine particularly if, as is often the case, glucosamine predominates. Traces of methyl esters of fatty acids which may occur in preparations of membrane glycoproteins are also liable to cause confusion in this area of the chromatogram unless previously removed by extraction with hexane [2].

Hydrolysis in *p*-toluenesulphonic acid at 100°C is therefore the preferred method as the recovery of hexosamines is about 95% in the 24 h hydrolysate which is almost within the range of error of the amino acid analyser. With the amino acid analyser glucosamine and galactosamine can be separated from each other and from the amino acids by a number of buffer systems; for glycoproteins which contain tryptophan,

elution with the pH 4.25 analyser buffer only should be used, because in other systems peaks due to tryptophan and its breakdown products are liable to coincide with hexosamines to give incorrectly high values. By the use of internal standards the hexosamine figures can be easily related to the g.l.c. analysis employed for neutral sugars and to the 110°C hydrolysate used for the analysis of the amino acids.

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