Further Studies on Gel Filtration of Urinary Substances[1]

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Summary. Urine specimens from healthy children were concentrated and filtered through Sephadex G-100 and G-25. The eluate was recovered in fractions of 5 ml each. Total hexoses, galactose, total proteins and total uronic acids were determined on each fraction. Total hexoses and galactose were eluted in 6 distinct peaks. The uronic acid containing GAG were eluted in 4 small peaks on G-25 and 1 peak on G-100. This peak is eluted always with high amounts of galactose containing glycoproteins.

Urinary mucosubstances and their derivatives have been studied by means of gel filtration on various Sephadex types[3,4]. In addition, several oligosaccharides containing fucose and sialic acids, as well as a few glycopeptides with glucose and galactose, have been isolated from normal urine and characterized[5,6]. Most of the papers published emphasized the study on the molecular weight range from 1,000 to 5,000 and gave no information on compounds of higher weight.

The purpose of the present investigation was to study the molecular weight distribution of glycopeptides and associated substances in normal urine. An attempt has been made also to establish the approximated molecular weights of some peaks.

Material and methods. Urine. Urine specimens of 24 h were collected from 6 male healthy children of 4.5 years $\pm$ 3 months of age and processed immediately. From each specimen, a sample of 20 ml was taken; it was filtered at 4 °C in order to remove insoluble matter. The urine was then concentrated 4-fold in a rotary vacuum evaporator at 40 °C.

Gel filtration. A column of 68×1.5 cm was prepared from Sephadex G-100. The void volume, as determined eluting a zone of Blue dextran, was found to be 25 ml. The concentrated sample was added to the column and the elution was performed with distilled water. The eluate was recovered in fractions of 5 ml each, the filtration rate being about 10 ml/h.

The void volume was then concentrated 5-fold and developed on a similar column of Sephadex G-25 fine

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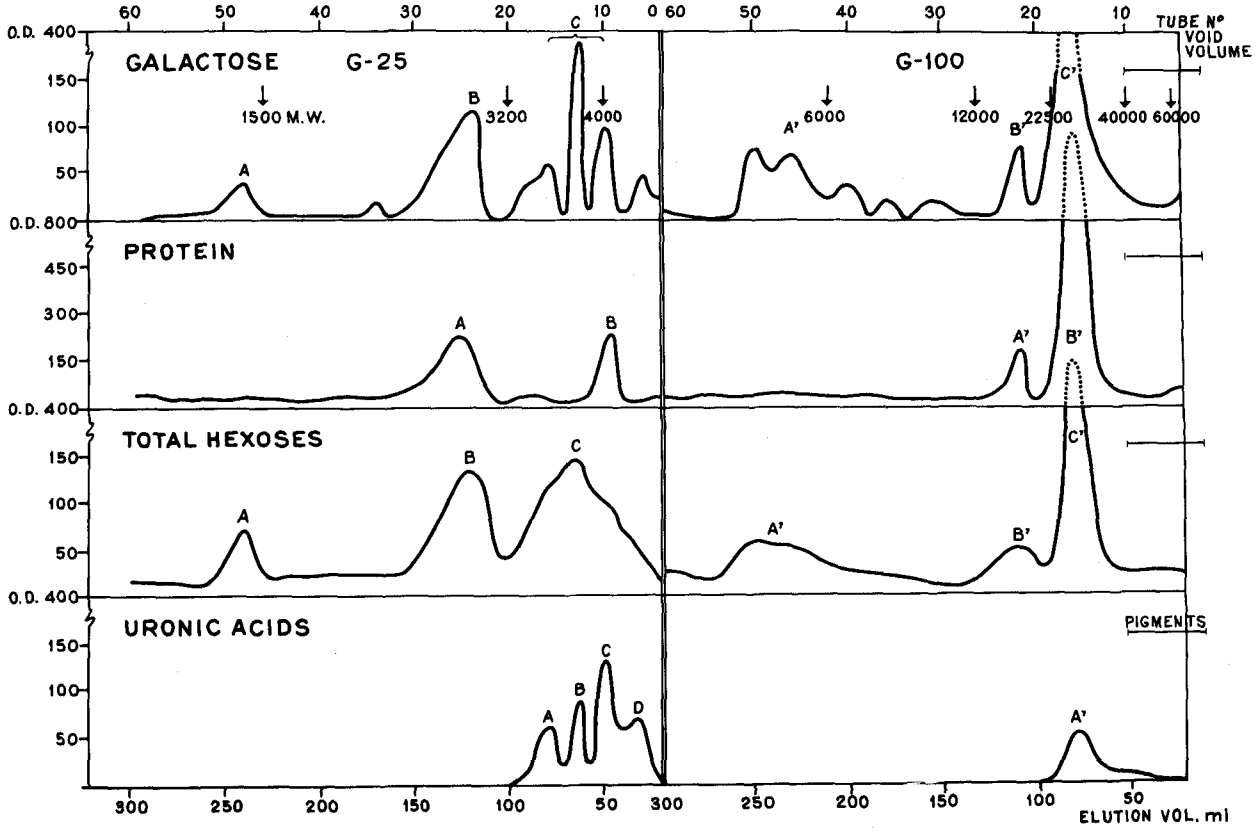
[3] A. Lundblad and I. Berggård, Biochim. biophys. Acta *57*, 129 (1962).

[4] J. S. Davis, F. V. Flynn and H. S. Platt, Clin. chim. Acta *21*, 357 (1968).

[5] T. A. Miettinen, Clin. chim. Acta *8*, 693 (1963).

[6] D. Basu, Biochem. J. *112*, 379 (1969).



with distilled water. The void volume was found to be 20 ml. The total elution volume for each sample was 300 ml. Purified polypeptides and proteins of known molecular weight were filtered under the same conditions in order to determine the elution volume.

Analysis. Total proteins were determined on an aliquot of the fractions by measuring the optical density at 280 nm and by the LOWRY-FOLIN method[7] using crystalline serum albumin as standard. Galactose was measured with the technique of DISCHE[8] using galactose Sigma as standard. Total hexoses were determined by the method of YEMM and WILLIS[9] using glucose as standard. Total uronic acids were measured by the carbazol method of BITTER and MUIR[10] using glucuronic acid as standard.

Results. The Figure shows the elution curves of the urine concentrates corresponding to 20 ml of filtered urine, on Sephadex G-100 and G-25. The line represents the average values of 6 samples, each of them run in duplicate. The void volume of Sephadex G-25, almost neglegible in most cases, was not represented. The column was eluted with distilled water. Pigments were normally reversibly adsorbed on the columns and appeared in the eluate as shown in the Figure.

In all experiments, authrone analyses gave 3 distinct peaks on both Sephadex G-100 and G-25. These results were in agreement with those of LUNDBALD et al.[3] Peak A varied in height in the different samples, and peaks B and C, always distinct, were almost constant in height. On Sephadex G-100 the small peaks A′ and B′ were not constant, but peak C′ appears in all samples with similar height. Probably most of the hexoses of these peaks were galactose, as can be seen in the corresponding elution curves.

The galactose curve approximately followed the total hexoses curve, with the exception of peaks C an G-25 and A′ on G-100 that are resolved in about 66% of the analyses in 3 peaks. Peak A on this curve has a MW near 1,500; peak B about 3,000 and peak C about 4,000. Peak A′ on Sephadex G-100 has a molecular weight about 6,000 and peaks B′ and C′ are near 22,000 MW.

The total proteins curve shows some similarity with that of total hexoses, but peaks A and A′ are absent. According with the above results, most of the protein would be galactose containing glycoproteins. This is in agreement with NORDEN's[11] findings on the galactose content of normal urine.

The uronic acid recorded on Sephadex G-25 is eluted in 4 peaks ranging from 3,500 to 4,000 MW. Peaks A and B were almost constant and distinct. Peaks C and D varied in height with the different samples. Microcolumn fractionation[11] and IR-spectra were used in order to identify the uronic acid containing glycosaminoglycans present in the fractions. Chondroitin-4-sulfate and heparitin sulfate were the glycosaminoglycans identified. Also traces of hyaluronic acid and heparin have been found. Probably all peaks represent degradation products of glycosaminoglycan-protein complexes of different molecular weights. Peak D was always eluted with a low galactose peak. The uronic acid recorded in G-100 appears in only one peak (A′) (M W about 25,000) being always eluted with galactose containing glycoproteins[12]. Work is in progress with further separation and closer chemical characterization of various fractions described in this paper.

[7] O. H. LOWRY, H. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).
[8] Z. DISCHE, in *Methods in Biochemical Analysis* (Ed. D. GLICK; Interscience, New York 1955), vol. 2, p. 313.
[9] E. W. YEMM and A. J. WILLIS, Biochem. J. *57*, 508 (1954).
[10] T. BITTER and H. M. MUIR, Analyt. Biochem. *4*, 330 (1962).
[11] N. E. NORDEN, Ö. ERIKSSON, B. HULTBERG and P. A. ÖCKERMAN, Clin. chim. Acta *44*, 95 (1973).
[12] J. SVEJCAR and W. B. VAN ROBERTSON, Analyt. Biochem. *18*, 333 (1967).

Repressible Alkaline Phosphatase in *Aspergillus niger*

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Summary. ALP from *A. niger* is a) P_i repressible enzyme; b) stimulated by addition of Zn^{++} to the growth medium, and c) that EDTA inhibits the enzyme reversibly, which could be restored by addition of Zn^{++} and perhaps Mg^{++}. This property is in contrast to the enzyme from *N. crassa*, which is independent of any metal requirement.

Alkaline phosphatase ALP[2] E.C. 3.1.3.1 occurs in *E. coli*[3,4] and in a number of other organisms[5–7]. The enzyme is non-specific, repressed by P_i, is a metalloprotein and requires Zn^{++} for its maximal activity[8]. ALP from *N. crassa*[5] has been separated into 2 components, one of which is repressed by P_i and the other derepressed. The repressed enzyme does not require any metallic ion for its activity but is stimulated by addition of EDTA. This study relates to a P_i repressible ALP from *A. niger*, which is similar to *E. coli* enzyme in its requirement for Zn^{++}. The enzyme is inhibited by both EDTA and 1:10 phenanthroline. The EDTA inhibition is reversed by Zn^{++}.

Aspergillus niger N.R.R.L. 67 was obtained from Northern Regional Research Laboratory, Peoria, Illinois, USA. The organism was grown on a modified Czapek-Dox medium. The modifications were that $NaNO_3$ was replaced by NH_4NO_3 and K_2HPO_4 by KH_2PO_4; 0.7 m*M* $ZnSO_4 7H_2O$ was added as a supplement to the medium unless otherwise noted, and pH adjusted to 2.2–2.3 with dilute HNO_3. Fungal mats grown under stationary culture at 28 °C for 96 h were washed thrice with ice-cold distilled

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[2] Abbreviations: ALP, alkaline phosphatase; EDTA, ethylene diamine tetra acetic acid disodium salt; *p*-npp, *p*-nitrophenyl phosphate; *p*-np, *p*-nitrophenol; P_i, KH_2PO_4; OD_{410}, optical density at 410 nm; SA, specific activity.
[3] T. HORUICHI, S. HORUICHI and D. MIZUNO, Nature, Lond. *183*, 1529 (1959).
[4] A. TORRIANI, Biochim. biophys. Acta *38*, 460 (1960).
[5] J. F. NYC, R. J. KADNER and B. J. CROCKEN, J. biol. Chem. *241*, 1468 (1966).
[6] T. C. STADTMAN, Biochim. biophys. Acta *32*, 95 (1959).
[7] F. P. HEALEY, J. Phycol. *9*, 383 (1973), consulted in Chem. Abstr. *81*, 35420 m (1973).
[8] D. J. PLOCKE, C. LEVINTHAL and B. L. VALLEE, Biochemistry *1*, 373 (1962).