13. Abadom, P. N. and Elson, D. *Biochim. Biophys. Acta* **199** (1970) 528.
14. Solymosy, F., Fedorcsák, I. and Ehrenberg, L. *Unpublished results.*
15. Fraenkel-Conrat, H. In Cantoni, G. L. and Davies, D. R. *Procedures in Nucleic Acid Research*, Harper and Row, New York 1966, p. 480.
16. von Ehrenstein, G. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*, Academic, New York 1967, Vol. 12 A, p. 592.

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Separation of Estriol Conjugates on Sephadex

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Beling [1] used gel filtration on Sephadex G-25 to separate estrogen conjugates in late pregnancy urine into two major fractions, referred to as peaks I and II. This method eliminates most of the urinary constituents that would otherwise interfere with enzymatic hydrolysis,[1,2] and separation of estrogen 3-glucuronides from estrogen 16-glucuronides is achieved. However, sulfates and sulfoglucuronides cannot be separated from glucuronides by this procedure. Methods to subfractionate peaks I and II by reapplication on various hydrophilic Sephadex columns have been described.[3,4] A gel chromatographic system in which the Sephadex G-25 column was eluted with an organic solvent has been used in the separation of various peak I and II conjugates.[1,5-7]

The advantages of a lipophilic gel, Sephadex LH-20, for the purification and separation of neutral steroid conjugates [8-11] have been demonstrated but its possible merits in the separation of conjugated estrogens have seemingly been overlooked. Accordingly, we set out to explore the potentialities of Sephadex LH-20 for the separation of peak I and II conjugates. This communication describes preliminary studies on a method to separate four urinary estriol conjugates by sequential gel filtration on Sephadex G-25 and chromatography on Sephadex LH-20. A similar scheme can probably be used for the fractionation of a number of other urinary estrogen conjugates as well.

Methods. Sephadex G-25 and Sephadex LH-20 were purchased from Pharmacia AB, Uppsala, Sweden. Gel filtration of urine specimens on Sephadex G-25 columns was performed according to Beling.[1] When reference compounds were gel-filtered in the absence of urine, the samples were dissolved in 10 ml of 0.1 M phosphate buffer, pH 6.5, containing 8 mg of uric acid, which led to elution of peak I and II conjugates in the correct position.[3] Flow rate was maintained at 0.2 ml/min with a peristaltic pump, and fractions were collected automatically. The gel chromatographic procedures were modified from a method used in the separation of urinary neutral steroid conjugates.[11] Columns of 4 g of Sephadex LH-20 (300 × 10 mm) were prepared in chloroform/methanol 1:1 containing 0.01 mol/l sodium chloride giving a bed height of approximately 25 cm. The samples were applied in 2–3 ml of the eluting solvent, which consisted of chloroform/methanol mixtures containing 0.01 mol/l sodium chloride, or, when higher percentages of chloroform were used, the eluent was saturated with sodium chloride (Ref. 9). Solvent flow rate was about 0.5 ml/min and fractions were collected automatically. Radioactivity was measured in a Wallac NTL 314 liquid scintillation spectrometer.

Labeled reference compounds. Four labeled estriol conjugates, estriol-3-glucuronide (E_3–3Gl), estriol-3-sulfate, 16-glucuronide (E_3–3S, 16Gl), estriol-16-glucuronide (E_3–16Gl) and estriol-3-sulfate (E_3–3S), were prepared biosynthetically from estriol-6,7-^{3}H and estriol-4-^{14}C (New England Nuclear Corporation, Boston, Mass., U.S.A.). Gel filtration on Sephadex G-25 in the presence of uric acid (see above) proved to be a convenient method for the initial separation of estriol conjugates after the biosynthetic procedure. Labeled E_3–16Gl, synthesized according to Slaunwhite *et al.*,[12] was converted to E_3–3S,16Gl by incubating in a 100 000 *g* supernatant of homogenized guinea-pig liver in the presence of ATP and $MgSO_4$ (Levitz *et al.*[13]). Gel filtration on Sephadex G-25 in urate-phosphate buffer gave the synthesized E_3–3S,16Gl (50–90 % of the radioactivity) in the frac-

tions corresponding to peak I and the unchanged E_3-16Gl in the fractions corresponding to peak II. After evaporation, the peak I fractions were incubated with 1000 U/ml of β-glucuronidase (Ketodase, Warner-Chilcott Laboratories, Morris Plains, New Jersey, U.S.A.) in 0.1 M acetate buffer, pH 4.5, for 16 h and refiltered through Sephadex G-25 in urate-phosphate buffer. In the peak II fractions, 97 % of the radioactivity was eluted and only traces were found in the peak I fractions, indicating almost quantitative cleavage of $E_3-3S,16Gl$ to E_3-3S. Labeled E_3-3Gl was prepared according to Goebelsman *et al.*[14]

Results and discussion. Gel filtration studies. When in separate experiments labeled reference compounds were added to 10 ml specimens of pregnancy urine and gel-filtered on Sephadex G-25 columns (500 × 10 mm),[1] sharp symmetrical peaks were obtained for $E_3-3S,16Gl$ (peak I) and for E_3-3S and E_3-16Gl (peak II). E_3-3Gl (peak I), however, was invariably eluted somewhat later than $E_3-3S,16Gl$ and gave a broader peak with incomplete separation from peak II conjugates. By increasing the column lengths with twice the amount of gel, an acceptable separation of peak I and II fractions was achieved.

Gel chromatographic studies. When applied on a Sephadex LH-20 column and eluted with chloroform/methanol 1:1 containing sodium chloride, the two labeled estriol monoglucuronides (E_3-3Gl and E_3-16Gl) were recovered in the effluent between 20 and 45 ml. The estriol monosulfate (E_3-3S) was eluted in the fractions between 40 and 65 ml. This closely resembles the elution of neutral steroid glucuronides and monosulfates, respectively, under similar experimental conditions.[11,15] The estriol double conjugate ($E_3-3S,16Gl$) was strongly retarded in the column and was not eluted in the first 150 ml of effluent. In subsequent experiments the elution of $E_3-3S,16Gl$ was facilitated by changing the eluent to methanol after elution of the "glucuronide" fraction. In all chromatographic experiments with Sephadex LH-20 free estriol was eluted in the first 15 ml of effluent.

Separation of labeled peak I conjugates on Sephadex LH-20. In the system described above, complete separation of E_3-3Gl and $E_3-3S,16Gl$ could be obtained. To eliminate slight tailing of E_3-3Gl to subsequent fractions, chloroform/methanol 2:3 containing sodium chloride (0.01 mol/l) was used as eluent in the final method. Under these conditions E_3-3Gl was recovered in the fractions between 20 and 40 ml. After elution of the column with 70 ml, the eluent was changed to methanol, which led to elution of $E_3-3S,16Gl$ in the effluent between 80 and 105 ml (Fig. 1).

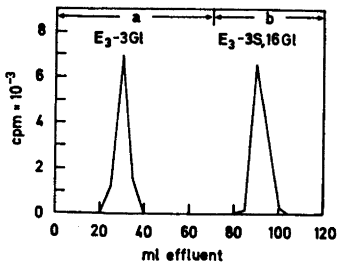

Fig. 1. Separation of labeled E_3-3Gl and $E_3-3S,16Gl$ on a 4 g Sephadex LH-20 column. Solvent: a, 0.01 M NaCl in $CHCl_3$:CH_3OH 2:3; b, CH_3OH.

Separation of labeled peak II conjugates on Sephadex LH-20. When chloroform/methanol 1:1 containing sodium chloride (0.01 mol/l) was used as eluent, overlapping of E_3-16Gl with E_3-3S occurred (see above). An increase in the proportion of chloroform caused retardation of both conjugates in the column, but E_3-3S was retained to a higher degree. With chloroform/methanol 2:1 (saturated with sodium chloride) as eluent, satisfactory separation of E_3-16Gl (40–80 ml), from E_3-3S (95–135 ml) was obtained (Fig. 2).

Separation of labeled estriol conjugates added to pregnancy urine. Labeled estriol

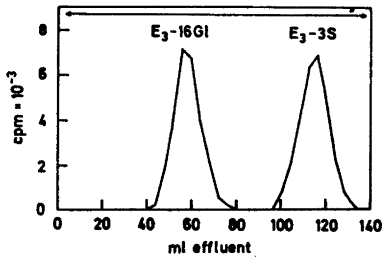

Fig. 2. Separation of labeled E_3-16Gl and E_3-3S on a 4 g Sephadex LH-20 column. Solvent: $CHCl_3$:CH_3OH 2:1, saturated with NaCl.

conjugates added to pregnancy urine were gel-filtered on Sephadex G-25, and peaks I and II were collected separately and evaporated to dryness *in vacuo*. The dry residues were taken up in 3 ml of the eluting solvent and chromatographed in the systems designed for peak I and II conjugates. The presence of urinary peak I and II contents in the samples did not alter the elution pattern of the labeled reference compounds.

Application. In chromatographic experiments on Sephadex LH-20 the elution pattern of labeled estriol glucuronides and estriol sulfate closely resembled that of the neutral steroid glucuronides and monosulfates. In addition, the estriol double conjugate (E_3—3S,16Gl) was strongly retarded in the column, as is the case with the neutral steroid disulfates.[11,15] This seems to confirm earlier studies,[8,9] which suggested that it is the mode of conjugation, rather than the steroid nucleus, which determines the elution behaviour of conjugated steroids on lipophilic Sephadex. Experiments with labeled estriol conjugates indicate that by chromatography on Sephadex LH-20 a convenient group separation of estrogen conjugates may be achieved, where at least glucuronides, sulfates, and sulfoglucuronides can be separated from each other. In addition, gel filtration on Sephadex G-25 can be used in the separation of estrogen 3-glucuronides from estrogen 16-glucuronides. Thus it can be concluded that gel chromatography on Sephadex LH-20 in combination with gel filtration on Sephadex G-25 provides a useful means for group separation of urinary estrogen conjugates.

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1. Beling, C. G. *Acta Endocrinol. Suppl.* **79** (1963).
2. Adlercreutz, H. *Acta Endocrinol.* **57** (1968) 49.
3. Smith, E. R. and Kellie, A. E. *Biochem. J.* **104** (1967) 83.
4. Paul, W., Stitt, C., Dignam, W. J. and Kushinsky, S. *J. Chromatog.* **45** (1969) 392.
5. Adlercreutz, H. *Scand. J. Clin. Lab. Invest. Suppl.* **86** (1965) 121.
6. Adlercreutz, H., Svanborg, A. and Ånberg, Å. *Am. J. Med.* **42** (1967) 341.
7. Tikkanen, M. J., Juusela, H. and Adlercreutz, H. *Scand. J. Clin. Lab. Invest. Suppl.* **113** (1970) 45.
8. Sjövall, J. and Vihko, R. *Acta Chem. Scand.* **20** (1966) 1419.
9. Vihko, R. *Acta Endocrinol. Suppl.* **109** (1966).
10. Laatikainen, T. *Ann. Clin. Res. Suppl.* **5** (1970).
11. Jänne, O. *Clin. Chim. Acta* **29** (1970) 529.
12. Slaunwhite, R. W., Jr., Lichtman, M. A. and Sandberg, A. A. *J. Clin. Endocrinol. Metab.* **24** (1964) 638.
13. Levitz, M., Katz, J. and Twombly, G. H. *Steroids* **6** (1965) 553.
14. Goebelsman, U., Diczfalusy, E., Katz, J. and Levitz, M. *Steroids* **6** (1965) 859.
15. Sjövall, J., Sjövall, K. and Vihko, R. *Steroids* **11** (1968) 703.

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Studies on Orchidaceae Alkaloids

XX.* The Constitution and Relative Configuration of Crepidine, an Alkaloid from *Dendrobium crepidatum* Lindl.

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Five closely related crystalline alkaloids have been isolated from *Dendrobium crepidatum* Lindl.[2] One of the bases ($C_{21}H_{29}NO_3$), named crepidine (I), crystal-

* No. XIX of this series, see Ref. 1.