

and evaporated. The crystalline substance obtained was recrystallized from benzene-cyclohexane. About 0.4 g (70 %) of needle shaped crystals, m.p. 47–48°, were thus obtained. For analysis see below.

2) *Synthesis in the presence of acetic acid.* 2.3 ml (40 mmole) of acetic acid dissolved in 25 ml of tetrahydrofuran were slowly added under stirring during 30 min to a mixture of 1.6 g (42 mmole) of sodium borohydride and 2.0 ml (39 mmole) of aziridine in 50 ml of tetrahydrofuran. The temperature was maintained below 25°. The reaction mixture was then stirred for 3 h under dry conditions, whereafter the precipitated sodium acetate and unreacted sodium borohydride were filtered off. The filtrate was evaporated to dryness and the crystalline residue was recrystallized from benzene-cyclohexane. Yield 1.6 g (73 %), m.p. 47–48°. (Found: C 42.0; H 14.3; N 24.8; B 19.0. Calc. for C_2H_8BN : C 42.2; H 14.2; N 24.6; B 19.0).

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Chromatography of Conjugated Steroids on Lipophilic Sephadex

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Methylated Sephadex has been used in the separation of various lipid soluble substances.¹⁻⁴ In the course of gas chromatographic-mass spectrometric studies of

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solvolizable 17-ketosteroids in human serum, chromatography on methylated Sephadex was found to be valuable for the purification of monosulfates of 17-ketosteroids.⁵ This communication describes preliminary results on the chromatographic behaviour of conjugated steroids and bile acids on methylated Sephadex.

Methods. Methylated Sephadex G-25 with a content of methoxyl groups of about 36 % was prepared as previously described.² Sephadex LH-20 was kindly supplied by Dr. B. Gelotte, Pharmacia, Uppsala, Sweden. The columns were made with 25 g methylated Sephadex or 8 g Sephadex LH-20. Chloroform/methanol, 1:1, containing different electrolytes was prepared by adding one volume of chloroform to a 0.02 M solution of the electrolyte in methanol. If turbid, the solution was filtered. The samples were dissolved in 2–3 ml of the solvent. Solvent flow rate was about 0.4 ml/min. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (¹⁴C) or a Frieske-Hoepfner well counter (²²Na). Steroids were determined by measuring the absorbancy at the absorption maximum of the sulfuric acid chromogen after 2 h in concentrated sulfuric acid.⁶ Elution volumes of the compounds were expressed relative to that of radioactive cholesterol added as a standard to all samples.

We are greatly indebted to Dr. J. F. Becker and Dr. W. Klyne for gifts of 17-ketosteroid glucuronides. Sulfates of 17-ketosteroids were prepared according to Kornel *et al.*⁷ Reference samples of the sulfates of androsterone (A) and dehydroepiandrosterone (D) were kindly supplied by Dr. W. Klyne.

Results and discussion. *Effect of salts on the elution of steroid conjugates.* When 0.1 µg–1.5 mg of ¹⁴C-labeled sodium dehydroepiandrosterone sulfate (D–S) was chromatographed on 25 g columns of methylated Sephadex in chloroform/methanol, 1:1, the conjugate appeared as a broad peak with a relative elution volume of 0.70–0.80. When added to serum which was subsequently extracted and chromatographed under the same conditions,⁵ the labeled D–S appeared as a narrow peak with a relative elution volume of about 1.50. It appeared possible that serum electrolytes were responsible for the later elution of D–S added to serum (see Refs. 8, 9). Therefore steroid conjugates and bile acid were chromatographed on methylated Sephadex using chloroform/methanol containing different electrolytes as the solvent. As shown in Table 1

Table 1. Relative elution volumes of some steroid and bile acid conjugates chromatographed on methylated Sephadex with chloroform/methanol, 1:1, containing different electrolytes.

Electrolyte	Steroid *						
	D-S	D-G	A-G	TC	GC	C	
LiOH	0.65	—	0.72	0.61	0.61	0.66	
LiCl	0.66	—	0.89	0.62	0.75	0.81	
NiCl ₂	0.66	—	—	0.61	0.77	0.89	
CoCl ₂	0.69	—	—	0.61	0.71	0.88	
AlCl ₃	0.70	—	0.55	0.61	0.53	0.53	
CaCl ₂	0.87	—	—	0.73	0.78	0.80	
NaOH	1.27	1.40	—	1.03	1.08	1.15	
NaCl	1.33	1.10	—	1.04	1.07	0.90	
CsCl	1.41	—	0.94	1.06	0.80	0.84	
KOH	—	—	—	1.38	1.37	1.42	
KCl	2.10	—	0.97	1.42	1.45	1.10	

* The sodium salts, 0.3–0.5 mg, were dissolved in the respective solvents. D-S and D-G = dehydroepiandrosterone sulfate and glucuronide, respectively; A-G = androsterone glucuronide; TC, GC, and C = taurocholate, glycocholate, and cholate, respectively.

the type of electrolyte used and the nature of the acidic group in the conjugated steroid influence the elution volumes of the steroid conjugates. The retention volumes of neutral steroids were not influenced by the electrolytes (D and A had a relative elution volume of 0.95). Monosulfates of a number of steroids had approximately the same relative elution volume as D-S in chloroform/methanol, 1:1, containing NaCl or KCl. Thus the structure of the steroid nucleus is of minor importance in determining the elution volume of monosulfates in these solvents.

Steroid disulfates were eluted considerably later than the monosulfates, having relative elution volumes of about 2.40 with chloroform/methanol, 1:1, containing NaCl. In the solvent containing KCl the relative elution volumes were larger than 6.

Salts of D-S with pyridine, piperidine, quinoline, quinine, and triethylamine were chromatographed in chloroform/methanol, 1:1. Except with the pyridinium salt broad asymmetric peaks with relative elution volumes of 0.75–0.88 were obtained. The pyridinium salt gave a symmetrical

peak with a relative elution volume of 0.88. The separation of cholesterol and the pyridinium and sodium salts of cholesteryl sulfate is shown in Fig. 1.

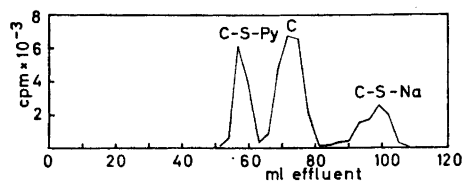


Fig. 1. Separation of cholesterol-4-¹⁴C (C) and the pyridinium (C-S-Py) and sodium (C-S-Na) salts of cholesteryl sulfate on a 25 g column of methylated Sephadex G-25 in chloroform/methanol, 1:1.

Ion exchanging properties of methylated Sephadex. The early elution of sodium D-S in chloroform/methanol without electrolyte was thought to be due to an uptake of Na⁺ by the methylated Sephadex. When 0.01 M sodium acetate/acetic acid was added to the solvent the relative elution volumes of D-S became smaller with an increasing proportion of acetic acid. When ²²Na₂CO₃ was applied to a column ²²Na was taken up by the methylated Sephadex. A column was loaded with ²²Na₂CO₃ and unlabeled sodium D-S was then applied to the column. The peak of D-S (measured with sulfuric acid) had a relative elution volume of 1.18. A peak of ²²Na appeared in the later part of the D-S peak and had a relative elution volume of 1.35, i.e. the same elution volume as sodium D-S from a column with NaCl in the eluent. This shows that methylated Sephadex is a cation exchanger. Its capacity is about 10 μequiv./g, i.e. the same as found for regular Sephadex.⁸ It is evident that the ion exchanging properties should be taken into consideration when regular or methylated Sephadex are used for the purification of steroid conjugates from biological materials.

Effect of solvent composition and type of Sephadex. When chloroform/methanol, 1:1, 2:1, and 4:1 saturated with KCl were used as solvents D-S had relative elution volumes of 2.10, 2.55, and 4.8, respectively. When the more polar Sephadex LH-20 (a 2-hydroxyethyl substituted Sephadex) was used, the relative elution volumes of sodium D-S and sodium dihydroxycop-

rostane disulfate were 2.90 and 12, respectively, in chloroform/methanol, 1:1, containing NaCl (*i.e.* larger than with methylated Sephadex). The retention volume of cholesterol was about the same as with methylated Sephadex. These results indicate that the elution volumes are determined by a partition between a stationary gel-solvent phase and a less polar mobile solvent phase.

When water was present in the sample applied to the column considerable changes in the relative elution volumes were noted. Thus, when sodium D-S was dissolved in 8 ml chloroform/methanol/water, 1:1:0.3, containing 0.01 M KCl and applied to an 8 g Sephadex LH-20 column eluted with chloroform/methanol, 1:1, containing 0.01 M KCl, the relative elution volume of the steroid conjugate was 1.12 as compared to 3.7 when no water was present in the sample. Intermediate water contents gave broad double peaks between the relative elution volumes 1.12 and 3.7.

Applications. Chromatography on methylated Sephadex should be a useful technique for the group separation of free and conjugated steroids and bile acids (Table 1). The method has been used to purify steroid sulfates in extracts of human serum.^{5,10,11} In this case it is not necessary to add an electrolyte to the solvent (see above). Phospholipids, triglycerides, esterified and free cholesterol, and free steroids are eluted before the steroid monosulfates.² Steroid disulfates are eluted in a later fraction.

When the technique is used for the purification of reaction mixtures in the synthesis of steroid sulfates the addition of electrolytes to the solvents is undesirable. However, when the columns were pre-washed with 0.01 M NaOH or NaCl in chloroform/methanol, 1:1 (200–300 μ equiv./25 g methylated Sephadex) followed by chloroform/methanol, 1:1 (50–100 ml/25 g) the same elution volume for mg amounts of sodium salts of steroid sulfates were obtained whether the solvent contained NaCl or not.

Recovery studies with labeled D-S and various unlabeled steroid monosulfates have shown that losses on columns of methylated Sephadex are negligible.^{5,10}

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Crystal Structure of the *trans* Form of 1,4-Aminomethylcyclohexanecarboxylic Acid

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One of the stereo-isomers of 1,4-aminomethylcyclohexanecarboxylic acid exhibits a strong antifibrinolytic activity, the other almost none.¹

The hydrobromides of both forms of the amino-acid have recently been examined by X-ray crystallographic methods, and it has been established that the "active" form is the *trans* isomer.^{2,3}

In order to obtain precise informations regarding the *trans* isomer of the amino-acid itself, a crystal structure analysis has been carried out.

The crystals are orthorhombic with lattice parameters