CHROM. 4408

SEPARATION OF CONJUGATED URINARY ESTROGENS ON COLUMNS OF SEPHADEX®

II. SEQUENTIAL SEPARATIONS*

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(Received September 29th, 1969)

SUMMARY***

Columns of DEAE-Sephadex were used to separate conjugated estrogens in urine (metabolites of [4-^{14}C]estradiol, administered intravenously) and reference standards. A scheme has been devised whereby many of the conjugated estrogens in urine may be separated by means of a sequence of three chromatographic procedures on columns of Sephadex G-25, DEAE-Sephadex and Sephadex G-15. The composite elution profile contains at least eighteen distinct peaks of radioactivity.

INTRODUCTION

Many procedures for the separation of conjugated estrogens have been described. BELING[2] used gel filtration on Sephadex to separate conjugated estrogens in late pregnancy urine into two major fractions. SMITH AND KELLIE[3] showed that urate retards the elution of peak II of BELING and that peak I with appropriate pre-treatment can be subdivided into peaks IA and IB on a second column of Sephadex. Additional separation on columns of ECTEOLA cellulose and Celite 535 and by paper and thin-layer chromatography led to the indirect identification of a number of conjugated estrogens in late pregnancy urine. HAHNEL and co-workers[4,5] used DEAE-Sephadex with a gradient of sodium chloride to separate a number of estrogen sulfates and glucuronides in pregnancy urine and some reference standards. Evidence for the identity of some of the separated estrogens was based on further fractionation by means of columns of alumina and by TLC and PC. JIRKU AND LEVITZ[6] separated a number of biliary and urinary metabolites of [6,7-^{3}H]estrone-[^{35}S]sulfate by means of the following techniques: partition chromatography on columns of Celite, adsorption chromatography on alumina, gel filtration on Sephadex G-10 with aqueous methanol as eluant, high-voltage electrophoresis, PC and TLC.

* Supported by United States Public Health Service Grants AM-09908 and RCDA 5K3-AM-31,321 (S.K.) and a grant from the Ford Foundation.

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*** See preceding paper (ref. 1) for abbreviations used here.

DICZFALUSY and co-workers[7] have made extensive use of countercurrent distribution to identify conjugated estrogens in many biological fluids and in a variety of clinical and experimental situations. As more information is acquired on the large number of conjugated estrogens present in urine it becomes increasingly apparent that convenient and efficient methods are needed which will permit the separation of all the conjugated estrogens in urine. One ultimate objective of such endeavors is to assess the potential clinical significance of each of the conjugated estrogens which is present.

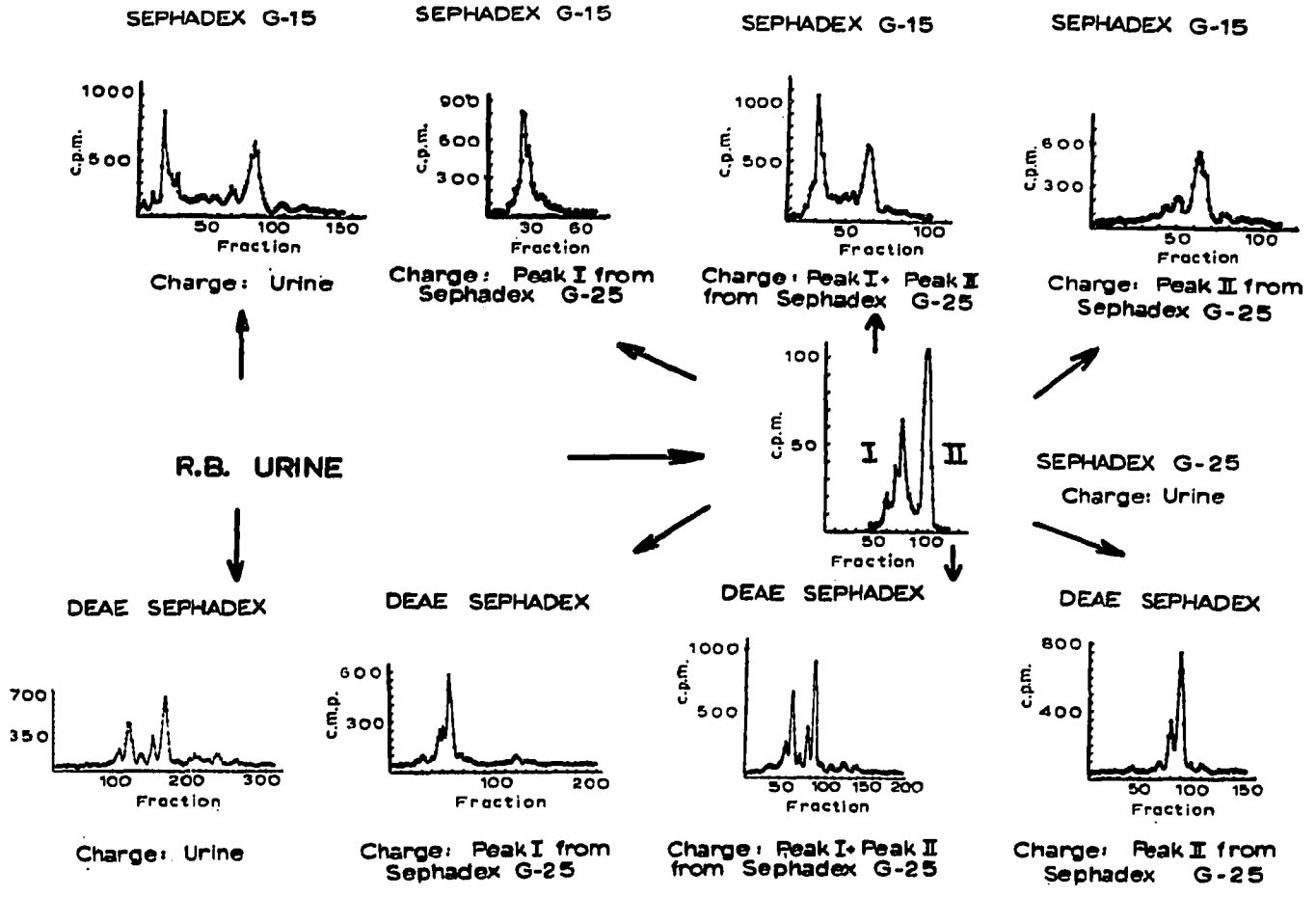

Fig. 1. Separation of conjugated estrogens in R. B. urine on columns of Sephadex G-15 and DEAE-Sephadex with and without prior separation on Sephadex G-25. Sephadex G-15 (0.9 × 100 cm): charge, peak I (0.5 ml) + peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 0.01 *M* ammonium formate; volume per fraction, 5 ml. Sephadex G-25 (6.5 × 180 cm): charge, R. B. urine (150 ml); eluant, distilled water; volume per fraction, 15 ml. DEAE-Sephadex (0.9 × 100 cm): charge, peak I (0.5 ml) + peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 1.0 *M* ammonium formate; volume per fraction, 3.3 ml. DEAE-Sephadex (0.9 × 100 cm): charge, peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 1.0 *M* ammonium formate; volume per fraction, 3.3 ml. DEAE-Sephadex (0.9 × 100 cm): charge, peak I (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 1.0 *M* ammonium formate, volume per fraction, 3.3 ml. Sephadex G-15 (0.9 × 100 cm): charge, peak I (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 0.01 *M* ammonium formate; volume per fraction, 5.0 ml. Sephadex G-15 (0.9 × 100 cm): charge, R. B. urine (1.5 ml); eluant, 0.01 *M* ammonium formate; volume per fraction, 2.5 ml. Sephadex G-15 (0.9 × 100 cm): charge, peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 0.01 *M* ammonium formate; volume per fraction, 5.0 ml. DEAE-Sephadex (0.9 × 100 cm): charge, R. B. urine (1.5 ml); eluant, 1.0 *M* ammonium formate; volume per fraction, 1.7 ml.

In the previous paper of this series[1] we described a series of studies in which conjugated estrogens in urine (metabolites of ^{14}C-labeled estradiol, administered i.v.) and reference standards were separated on columns of Sephadex G-10, G-15, and G-25 using various solutions of salts as eluants. In this paper are described studies on the separation of conjugated estrogens in urine using columns of DEAE-Sephadex A-25, alone and in conjunction with columns of Sephadex G-25 and Sephadex G-15. The resolving power of each system was evaluated in the light of the number of peaks of radioactivity obtained, the ease of execution of the column, the capacity of the columns and the amenability of the eluates to further fractionation. As a result of these studies a convenient scheme has been devised whereby a sequence of these chromatographic separations on columns of Sephadex G-25, DEAE-Sephadex and Sephadex

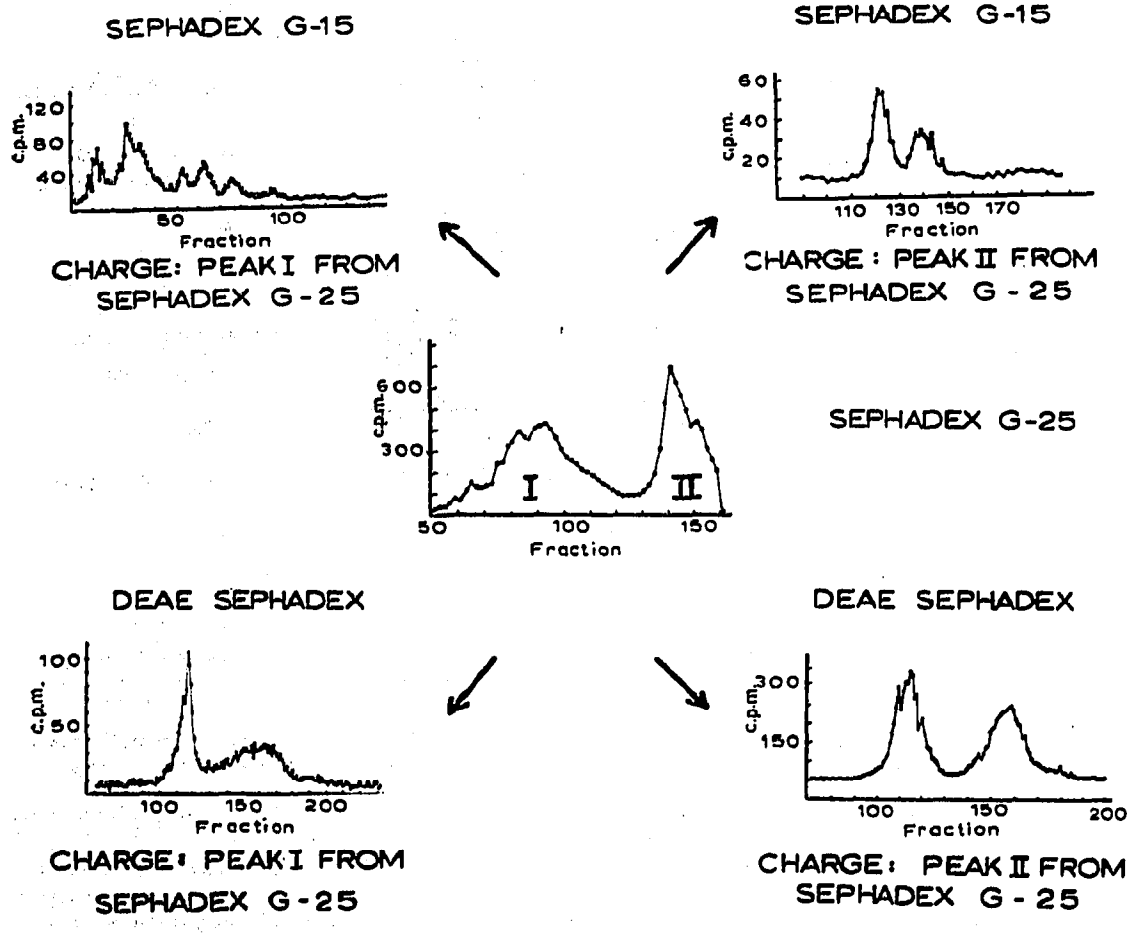

Fig. 2. Separation of conjugated estrogens in M. S. urine on columns of Sephadex G-15 or DEAE-Sephadex after prior separation on Sephadex G-25. DEAE-Sephadex (1.8 × 50 cm): charge, peak I (5.0 ml) from Sephadex G-25 (M. S. urine); eluant, linear gradient of 0.125 *M* ammonium formate (600 ml) to 0.30 *M* ammonium formate (600 ml); volume per fraction, 5.0 ml. DEAE-Sephadex (1.8 × 50 cm): charge, peak II (10 ml) from Sephadex G-25 (M. S. urine); eluant, linear gradient of 0.125 *M* ammonium formate (600 ml) to 0.30 *M* ammonium formate (600 ml); volume per fraction, 4.2 ml. Sephadex G-15 (2 × 100 cm): charge, peak I (5.0 ml) from Sephadex G-25 (M. S. urine); eluant, 0.2 *M* ammonium formate (pH 6.6); volume per fraction, 15 ml. Sephadex G-15 (2 × 100 cm): charge, peak II (5.0 ml) from Sephadex G-25 (M. S. urine); eluant, 0.2 *M* ammonium formate; volume per fraction, 3.5 ml. Sephadex G-25 (6.5 × 155 cm): charge, M. S. urine, day 2 (150 ml); eluant, distilled water; volume per fraction, 20 ml.

G-15 provides at least eighteen distinct peaks of radioactivity. The scheme and some of the experiments which led to its development are described in this paper.

EXPERIMENTAL

Comparison of separation of conjugated urinary estrogens using columns of Sephadex G-15, Sephadex G-25 and DEAE-Sephadex

An aliquot of urine containing ^{14}C-labeled conjugated estrogens (R. B. urine, day 1 plus 2) was applied to a column of DEAE-Sephadex and eluted with 1.0 *M* am-

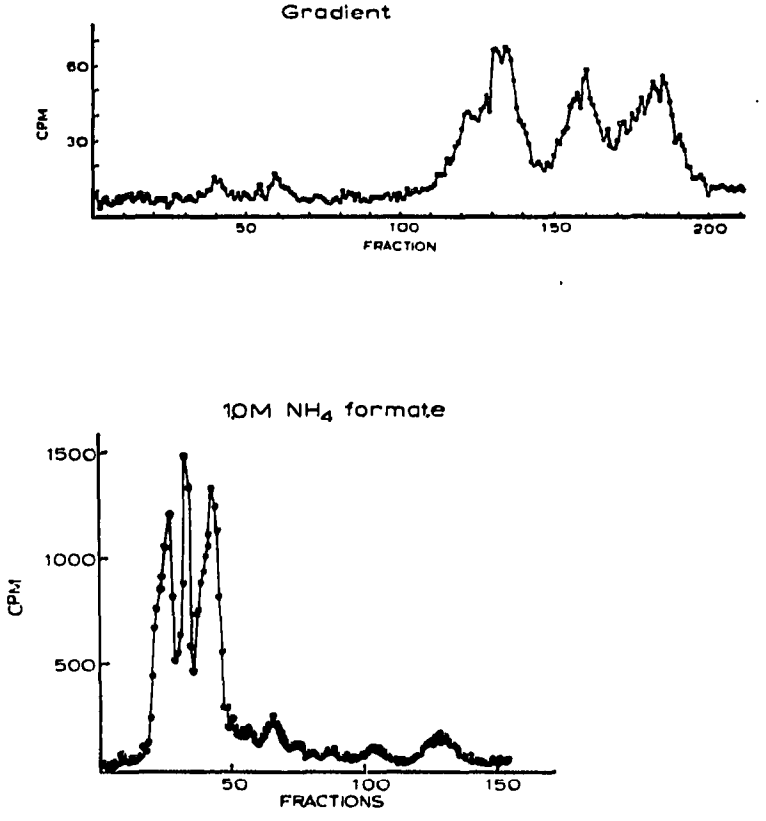

Fig. 3. Comparison of separation on a column of DEAE-Sephadex eluted with 1.0 *M* ammonium formate or with a gradient of ammonium formate. Upper part: linear gradient of 0.125 *M* ammonium formate (600 ml) to 0.25 *M* ammonium formate (600 ml): column dimensions, 1.8 × 50 cm; charge, M. S. urine (10 ml); volume per fraction, 10.5 ml. Lower part: 1.0 *M* ammonium formate; column dimensions, 0.9 × 100 cm; charge, M. S. urine (1.5 ml); volume per fraction, 4.8 ml.

monium formate. Other aliquots were applied to columns of Sephadex G-15 and Sephadex G-25 and eluted with 0.01 *M* ammonium formate and water, respectively. The elution patterns are shown in Fig. 1. At least nine distinct peaks are discernible in the elution patterns for DEAE-Sephadex and Sephadex G-15 compared with only two major peaks for Sephadex G-25. Peaks I and II from Sephadex G-25 were concentrated *in vacuo* and applied individually and together to columns of Sephadex G-15 and DEAE-Sephadex. The results of this study are summarized in Fig. 1. The overall resolution obtained with and without preliminary fractionation on Sephadex G-25

was not significantly different. There is a general trend for the components of peak I to be eluted from columns of DEAE-Sephadex and Sephadex G-15 before those of peak II.

In another study a different specimen of urine (M.S.) was separated on a column of Sephadex G-25 and peaks I and II were applied separately to columns of Sephadex G-15 and DEAE-Sephadex. The eluant used with the DEAE-Sephadex column was a linear gradient of ammonium formate from 0.125 *M* to 0.30 *M* (pH 6.6). Under these conditions (gradient) there is a clear advantage to preliminary separation on Sephadex G-25 since the elution volumes of peaks I and II are nearly identical on DEAE-Sephadex. The results are summarized in Fig. 2. In another study a comparison was made between the elution pattern from columns of DEAE-Sephadex (M. S. urine) using 1.0 *M* ammonium formate and a gradient of 0.125 *M* to 0.25 *M* ammonium formate (pH 6.6). The results are shown in Fig. 3. Although the elution volumes were larger when a gradient was used the resolution obtained is not significantly better than that found without the gradient and the gradient is less convenient to use.

Separation of conjugated urinary estrogens from the bulk of other substances on the basis of weight and radioactivity

A specimen of concentrated urine containing ^{14}C-labeled conjugated estrogens was applied to a column of DEAE-Sephadex (0.9 × 100 cm) and eluted with 1.0 *M* ammonium formate. Aliquots were removed for determination of the weight of the residue on evaporation to dryness. The results of this study, summarized in Fig. 4, show that there is almost a complete separation of the major weight-contributing urinary components from the conjugated estrogens. The relatively poor separation is a reflection of the large volume of eluant per fraction.

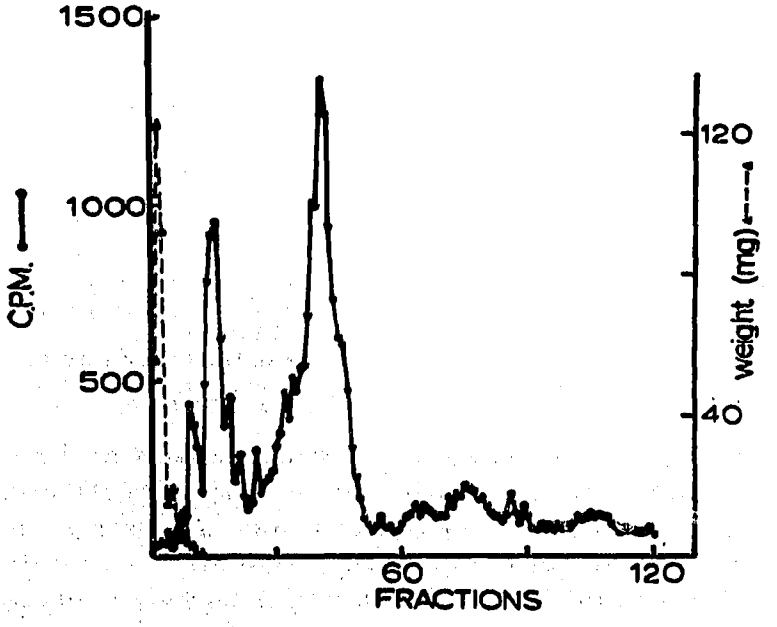

Fig. 4. Separation of conjugated urinary estrogens from the bulk of other substances on the basis of weight and radioactivity using a column of DEAE-Sephadex (0.9 × 100 cm). Charge, R. B. urine (4.0 ml); eluant, 1.0 *M* ammonium formate; volume per fraction, 3.0 ml.

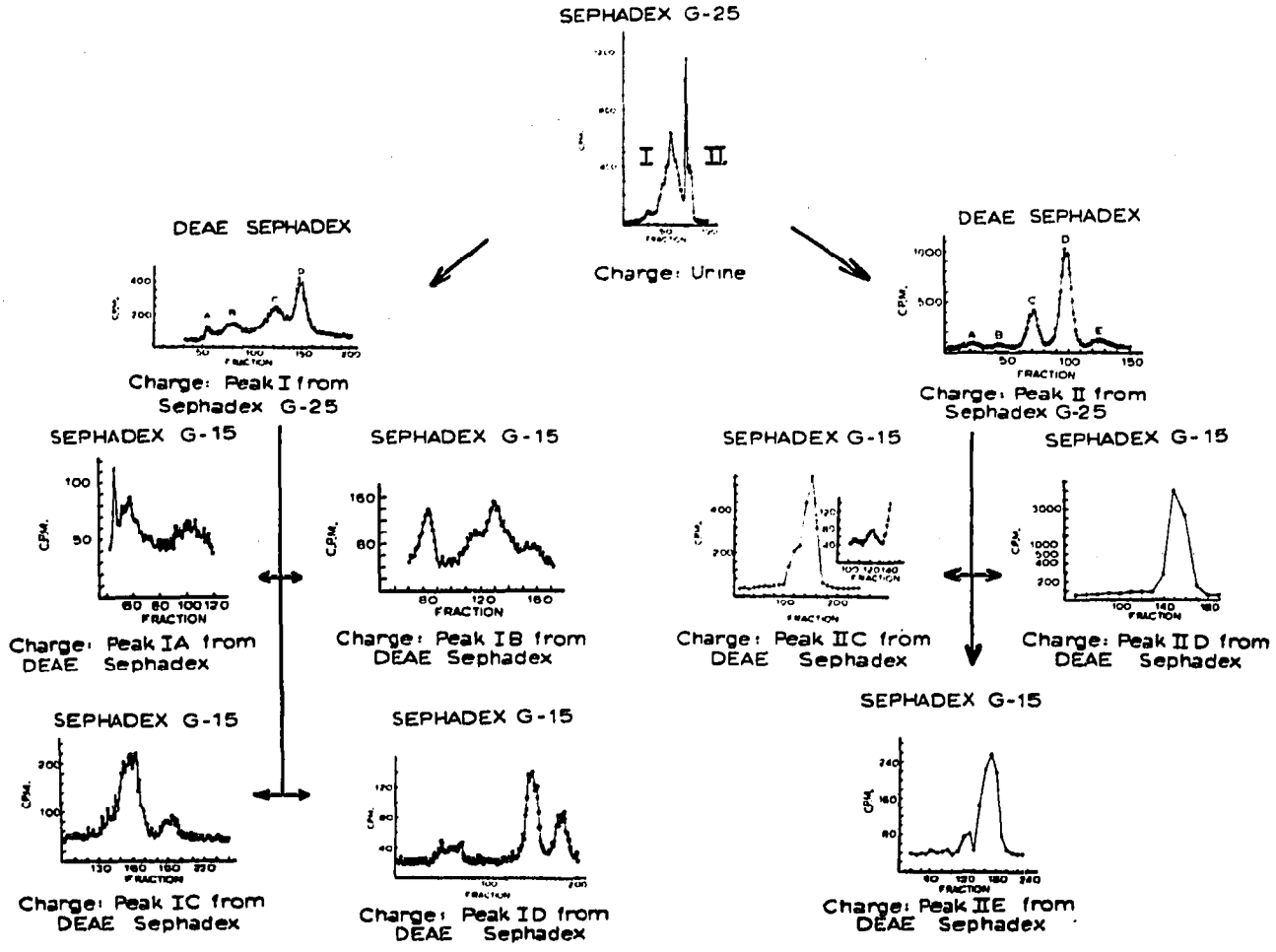

Fig. 5. Sequential separation of conjugated urinary estrogens on columns of Sephadex G-25, DEAE-Sephadex and Sephadex G-15. Sephadex G-25 (5 × 106 cm); charge, E. F. urine, days 2 + 3 (200 ml); eluant, distilled water; volume per fraction, 16 ml. DEAE-Sephadex (4 × 156 cm): charge, peak I (90 ml) from Sephadex G-25; eluant, 1.0 *M* ammonium formate; volume per fraction, 16 ml. DEAE-Sephadex (4 × 100 cm): charge, peak II (36 ml) from Sephadex G-25; eluant, 1.0 *M* ammonium formate; volume per fraction, 16 ml. Sephadex G-15 (2 × 200 cm): charge, peaks from DEAE-Sephadex (dry Sephadex G-15 added to solution of charge; mixture evaporated to dryness and applied to top of column); eluant, 0.01 *M* ammonium formate; volume per fraction, 13 ml.

Sequential separation of conjugated urinary estrogens on columns of Sephadex G-25, DEAE-Sephadex and Sephadex G-15

A specimen of concentrated urine containing ^{14}C-labeled conjugated estrogens was applied to a column of Sephadex G-25 and eluted with distilled water. The two primary fractions which were obtained (peaks I and II) were reapplied, after reduction of the volume *in vacuo*, to columns of DEAE-Sephadex and eluted with 1.0 *M* ammonium formate. Peak I was resolved into four fractions (peaks IA, IB, IC and ID) and peak II into five fractions (peaks IIA, IIB, IIC, IID and IIE). Each of these fractions (except IIA and IIB which were too small for further fractionation) was applied to a column of Sephadex G-15 and eluted with 0.01 *M* ammonium formate. The elution pattern for each column is shown in Fig. 5. The scheme provided separation of at least eighteen different components as evidenced by the presence of eighteen dis-

crete or nearly discrete peaks. The pattern of IIC on Sephadex G-15 shows one peak and a pronounced shoulder which emerges as two distinct peaks when individual fractions were plotted (see inset) rather than pools of every ten fractions.

Elution volume from DEAE-Sephadex of reference standards in the presence and absence of urine

Four radioactive reference standards, estriol-3-glucuronide, estriol-16-glucuronide, estradiol-17-glucuronide and estrone-3-sulfate were applied to columns of DEAE-Sephadex in different combinations in the presence and in the absence of concentrated urine. The standards in the absence of urine were applied to columns with dimensions 0.9 × 50 cm and those in the presence of urine to columns with dimensions 0.9 × 100 cm. The elution volumes, summarized in Table I, are all different and each

TABLE I

ELUTION VOLUME OF REFERENCE STANDARDS IN THE PRESENCE AND ABSENCE OF URINE

Column, DEAE-Sephadex; eluant, 1.0 *M* ammonium formate. Reference standard: E_3-3-GA = estriol-3-glucuronide; E_3-16-GA = estriol-16-glucuronide; E_2-17-GA = estradiol-17-glucuronide; E_1-3-SO_4 = estrone-3-sulfate.

Column No.	*Column dimensions (cm)*	*Charge*	*Elution volume (ml)*			
			E_3-3-GA	*E_3-16-GA*	*E_2-17-GA*	*E_1-3-SO_4*
1	0.9 × 50	Standards only	91			
2	0.9 × 50	Standards only		118		
3	0.9 × 50	Standards only		120	147	
4	0.9 × 50	Standards only	88	127		307
5	0.9 × 50	Standards only		127		
6	0.9 × 50	Standards only	80		151	
7	0.9 × 50	Standards only			160	
8	0.9 × 50	Standards only			156	
9	0.9 × 100	Standards + urine		258		838
10	0.9 × 100	Standards + urine			296	1100
11	0.9 × 100	Standards + urine				800

of the standards is completely or nearly completely separated from the others. The elution volumes of the glucuronides were reproducible and were unaffected by the presence or absence of urine and appear to be related to the length of the column. There was considerably more variability in the elution volume of estrone sulfate but the elution volumes were consistently higher than those for any of the glucuronides studied.

DISCUSSION

Gel filtration on Sephadex G-25 with water as the eluant has been used to separate conjugated estrogens in urine from many other substances such as salts and inhibitors of β-glucuronidase which are present in urine[1]. The technique in addition separates completely and efficiently two major groups of conjugated estrogens on the basis of properties which are not likely to lead to separation by other chromatographic means. The capacity of the system, the relative ease of execution of the technique

and the benignity of the conditions are particularly favorable for its utilization as the first step in a scheme for the separation or isolation of conjugated estrogens in urine.

In the scheme which we have developed gel filtration on Sephadex G-25 is used as the first step and is followed by further fractionation on columns of DEAE-Sephadex and Sephadex G-15. It is apparent that under the conditions employed the factors operative in the separation of the conjugated estrogens are at least partially unique for each of the chromatographic systems which were used. Were this not so there would have been no additional resolution on sequential chromatographic manipulations. One specific example of a pair of standards which were separated on DEAE-Sephadex but not on Sephadex G-15 is estradiol-17-glucuronide and estrone-3-sulfate.

The separations achieved on columns of DEAE-Sephadex provided nine peaks of radioactivity from the components in the two peaks which were applied to the columns. In other studies, not described in the experimental section, sodium chloride was found to yield separations similar to those obtained with ammonium formate. The elution volumes were about 15% smaller with sodium chloride as the eluant and with the DEAE-Sephadex in the chloride form. However, since sodium chloride is more difficult to remove from the eluates once the separations have been achieved, ammonium formate was the preferred eluant.

In the preceding paper of this series[1] separation of conjugated urinary estrogens on columns of Sephadex G-15 under a variety of conditions was described. In this paper the technique was extended for use in separating further fractions already separated on columns of Sephadex G-25 and DEAE-Sephadex. As a result of the combined use of all three systems at least eighteen peaks of radioactivity were apparent in the elution patterns. It seems likely that additional fractionation by TLC on silica gel would provide sufficient additional fractionation for the essentially complete separation of the major conjugated estrogens in urine for structural analysis and ultimately for potential clinical use. Although no definitive identifications have been made there is evidence based on elution volumes for the presence in urine of estradiol-17-glucuronide, estriol-16-glucuronide and estriol-3-glucuronide but no evidence for the presence of estrone-3-sulfate. Based on the elution volume of estrone-3-sulfate from columns of DEAE-Sephadex (Table I) it is possible that in the sequence of chromatographic columns where eighteen peaks of radioactivity were obtained the estrogen sulfates were not eluted from the columns and that additional peaks would have been present if the elution had been prolonged to include the small amount of radioactivity which remained on the columns.

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