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COMBINATION OF CONVENTIONAL AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES FOR THE ISOLATION OF SO-CALLED "URAEMIC TOXINS"

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SUMMARY

Using fluids from the artificial kidney as an example, a generally useful combination of separation techniques is described for the preparative isolation of biologically active subfractions from extremely heterogeneous and diluted biological fluids. Haemofiltrate (20 l) and dialysate (100 l), respectively, are desalted and concentrated in one step by reverse osmosis using membranes with a nominal cut-off of 500 Daltons. The retentate with high concentrations of "uraemic toxins" is fractionated by preparative ion-exchange chromatography (double column technique with detection at 206 nm) and size exclusion chromatography yielding large amounts of ninhydrin-positive subfractions which inhibit DNA synthesis of rat bone marrow and HeLa cells *in vitro*, respectively. These fractions were analyzed by reversed-phase and size exclusion high-performance liquid chromatography. Many of the isolated fractions were shown to contain peptides.

INTRODUCTION

Chronic renal failure leads to a self-intoxication called "uraemia" and finally to death, unless regular dialysis treatment is started. The blood composition of these patients is characterized by high concentrations of numerous substances, which are not present or detectable only in trace amounts in normal sera. To these substances belong the so-called "uraemic toxins" including different chemical classes with widely differing molecular weights[1]. Previous membrane separation studies led to the hypothesis that, in addition to low-molecular-weight solutes, substances with molecular weights between 300 and 5000 Daltons, the so-called "middle molecules", and even higher-molecular-weight substances might play a part in uraemic intoxication[2]. The extreme heterogeneity and the small amounts of uraemic sera available for preparative studies might be responsible for the fact that the isolation of uraemic toxins in the middle- and higher-molecular-weight ranges was possible only in a few cases, and usually in very small amounts[3–10]. The isolation of pure substances, however, is important for chemical characterization and biological tests, which are often very sensitive to contaminations. Therefore, we have elaborated a general methodology for the preparative isolation of a broad range of biologically active substances from

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extremely heterogeneous and diluted biological fluids, in this case from fluids of the artificial kidney as an example. Membrane separation techniques were combined with conventional and high-performance liquid chromatography (HPLC) on analytical and preparative scales, yielding subfractions in high amounts for further characterization and biological testing.

MATERIALS AND METHODS

Reverse osmosis of haemofiltrates (from the PAN-15 and URF artificial kidneys, respectively) was performed using cellulose acetate membranes with a nominal cut-off of 500 Daltons (De Danske Sukkerfabrikker, Nakskov, Denmark) as described previously[11].

Ultrafiltration with a Millipore PTGC cassette was carried out for haemofiltrate preconcentration from 20 to 2 l, then diluted in distilled water to 5 l. The retentate was concentrated to 1 l and lyophilized (nominal cut-off of membranes: 10,000 Daltons).

Preparative ion-exchange chromatography was performed using the double column technique of Fürst *et al.*[12] with some modifications: DEAE-Sephacel, DEAE-Sepharose (Pharmacia) and DEAE-Trisacryl M (LKB); K50/30 columns at 5°C (Pharmacia); gel bed height 23 cm. The buffer leaving the mixing chamber of LKB-11300-Ultrograd was divided into two equal streams by a T-piece and two calibrated peristaltic pumps (LKB 2120) which were placed before the sample and reference columns, respectively. The eluates of the two columns were fed into the corresponding cuvettes (optical pathlength, 0.5 mm; volume, each 30 μ) of the dual channel photometer Uvicord III (LKB). The columns were eluted in parallel with a concentration gradient of NH_4HCO_3 at pH 8.6 as shown in Fig. 6.

Conventional size exclusion chromatography using Sephadex G-15 was performed as described in the figures and ref. 11.

High-performance reversed-phase liquid chromatography (HPLC) was performed with a Varian chromatograph 5020 equipped with a Vici-Valco automatic loop injector. A Vari-Chrom detector with 8-μl cell was used. Temperature: ambient. For further details see figures.

High-performance size exclusion chromatography was performed with a LKB-2150-HPLC pump, Rheodyne loop injector, LKB 2135-TSK G2000SW column (600 × 7.5 mm) and LKB 2238 Uvicord SII (8-μl cell). Temperature: ambient. For further details see figures.

In order to study the inhibitory activity of the isolated fractions of the DNA synthesis of rat bone marrow and HeLa cells *in vitro*, respectively, the test fractions were dissolved in the incubation medium of the cells and the incorporation rate of [^{3}H]thymidine into the acid insoluble fraction of the cells was measured (microtitre technique). For details see figures and ref. 13.

RESULTS AND DISCUSSION

The sera of patients in final stage renal failure show characteristic differences in their molecular weight distributions in comparison with normal sera (Fig. 1). Even a single dialysis treatment reduces these differences but is unable to normalize the

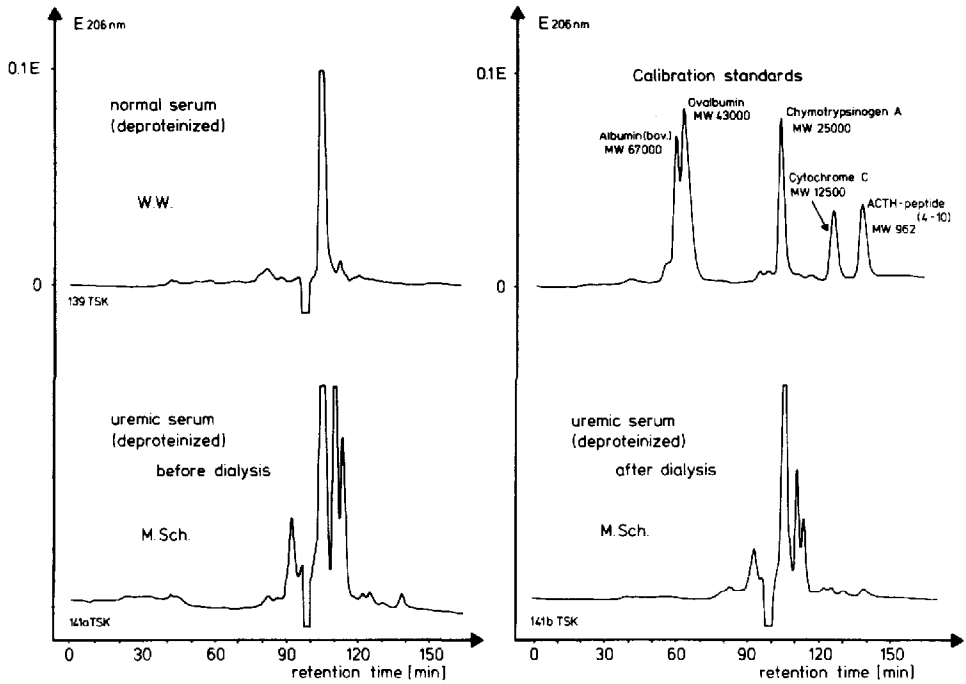

Fig. 1. Size exclusion chromatography of deproteinized (CF-25 Amicon cones) normal serum, uraemic sera before and after dialysis (each 20 μl) and calibration standards (10 μl containing 4–7 μg of each protein). Column: TSK G2000SW (LKB 2135) (600 × 7.5 mm). Eluent: 0.03 mol/l ammonium acetate; flow-rate 0.2 ml/min. W.W.: initials of healthy person; M.Sch.: initials for patient with end-stage renal failure. Absorption units for full-scale recorder deflection: 0.1E.

elution patterns completely. The differences are based not only upon the different molecular weight distributions, but also upon the widely differing polarities of the constituents, as can be deduced from reversed-phase chromatography (Fig. 2). The solutes removed from serum by regular dialysis treatment can be isolated from the dialysate or haemofiltrate, if it is possible to concentrate these relatively large volumes quickly and to desalt them by methods which don't destroy the biological activity of interesting fractions. A modified reverse osmosis with membranes having a nominal cut-off of 500 Daltons yields middle- and higher-molecular-weight fractions in high concentrations (Fig. 3) while more than 95% of the electrolytes are removed.

As we have shown earlier, some of the uraemic fractions have molecular weights above 10,000 Daltons[14]. Therefore, these special fractions were isolated by two methods: (a) dialysis of haemofiltrate in Visking tubes; (b) ultrafiltration of haemofiltrate using polysulphone membranes with a nominal cut-off of 10,000 Daltons (Millipore PTGC).

When these retentates were added to the incubation medium of rat bone marrow cells and HeLa cells *in vitro,* respectively, the [^{3}H]thymidine uptake of the cells was decreased by similar extents (Fig. 4). Also, these higher-molecular-weight fractions show extreme heterogeneity (Fig. 5), which may explain the relatively large amounts added to the cultures.

For systematic studies of a broad range of toxic solutes in the retentate from

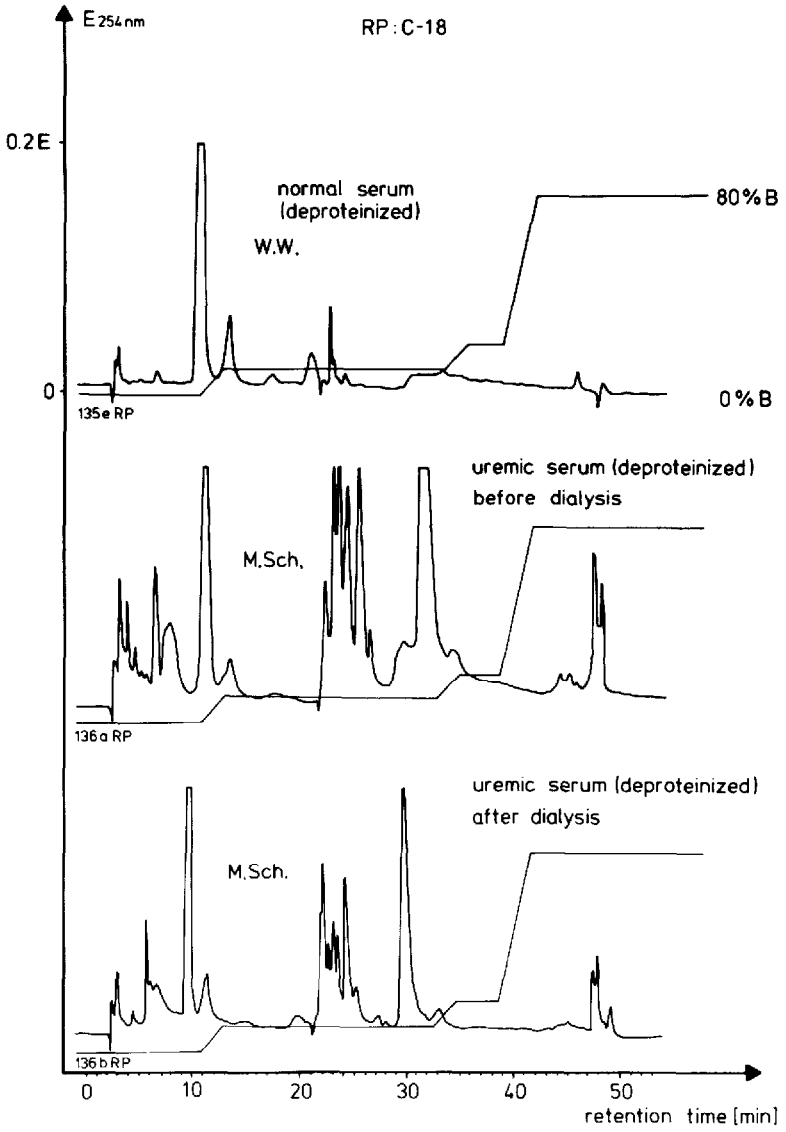

Fig. 2. Reversed-phase chromatography of deproteinized (CF-25 Amicon cones) normal serum and uraemic sera before and after dialysis, respectively (100 μl injections). Column: Varian-MCH-5-N-Cap (C_{18}), 150 × 5 mm, 5 μm. Eluents: A, 0.05% (v/v) TFA in water; B, 0.05% (v/v) TFA in CH_3CN; gradient as indicated. Flow-rate: 0.5 ml/min.

reverse osmosis, preparative ion-exchange chromatography was performed on different DEAE exchangers using the double column technique described by Fürst *et al*[12] and modified for macropreparative analyses (see Materials and Methods). This method enables monitoring of the eluate fractions at 206 nm even during gradient elution with strongly absorbing buffers (Fig. 6).

In spite of some disadvantages, we used the ammonium bicarbonate system throughout all conventional preparative fractionations, including also ion-exchange

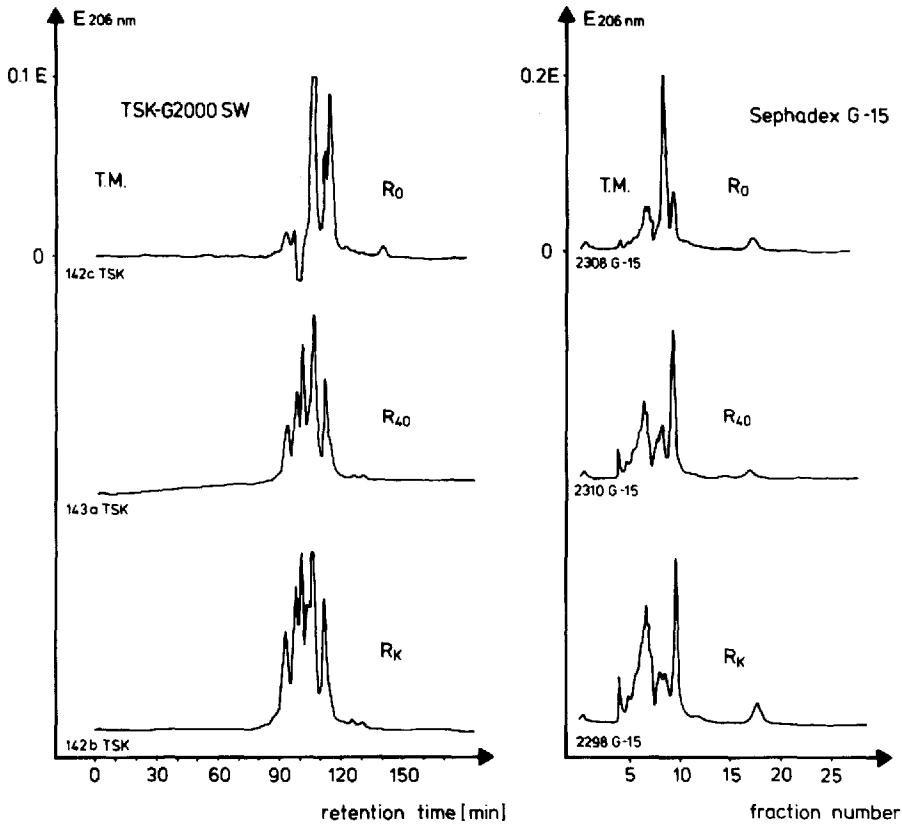

Fig. 3. Enrichment of uraemic toxins during reverse osmosis of 20 l haemofiltrate as shown by size exclusion chromatography of retentates at the beginning (R_0), after preconcentration and washing with 40 l (R_{40}) and after washing with 60 l distilled water and final concentration to 2 l (R_K), respectively. Left: 20 μl of each retentate injected onto a TSK G2000SW column (LKB 2135); conditions as in Fig. 1. Right: 1 ml of each retentate onto a Sephadex G-15 column, 200 × 1.1 cm; 0.05 mol/l NH_4HCO_3; linear flow-rate 75.2 cm/h (18.7 min per fraction).

chromatography, as it is volatile after repeated lyophilizations and interferences with biological test systems may be lower than with other buffers, which have been discussed previously[1,15]. The extreme heterogeneity based upon the different net charges at pH 8.6 is typical of the preparations from uraemic patients, whereas corresponding peaks from healthy persons are much smaller or even absent.

Each fraction was preparatively refractionated on Sephadex G-15, giving broad molecular weight distributions. As an example, the elution curves of the subfractions A, E, F and K, respectively, are shown in Fig. 7. The fact that the heterogeneity is additionally based upon the different polarities is obvious from Fig. 8. Even in fractions with a relatively narrow range of molecular weights and charge distribution, heterogeneity was evidenced by reversed-phase chromatography (Fig. 9).

Regarding the heterogeneity of haemofiltrate, it is clear that biological tests with unfractionated sera of patients cannot easily be interpreted. When the elution

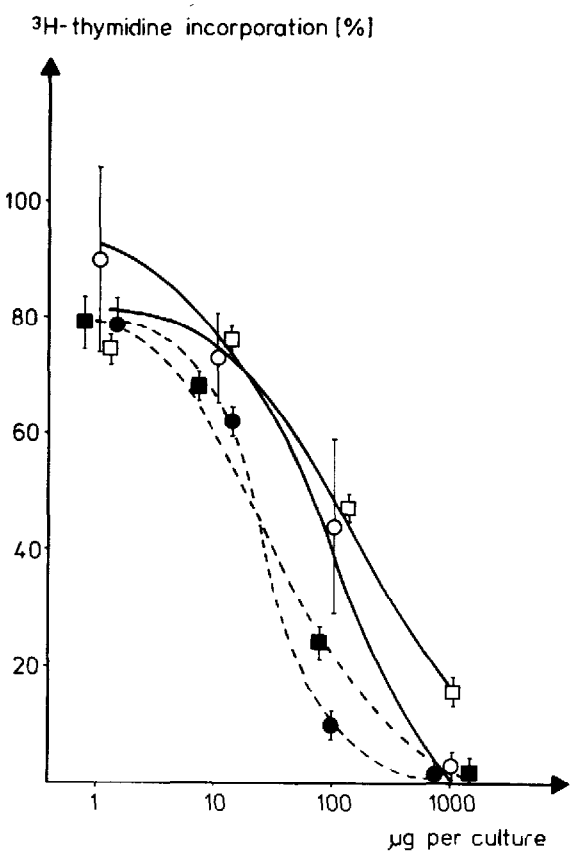

Fig. 4. Inhibition of [^{3}H]thymidine incorporation into rat bone marrow cells *in vitro* and HeLa cells *in vitro*, respectively, by retentates isolated from haemofiltrate using different membranes. All values are expressed as a percentage of incorporation into cultures without addition (= 100%). Means and ranges from five cultures are indicated. Retentates: from Millipore PTGC, HeLa (T.M.) (○—○) and bone marrow cells (T.M.) (□—□); from Visking tube dialysis, bone marrow cells (T.M., ●—●; J.B., ■—■); T.M. and J.B. are initials of two patients on regular haemodialysis).

profiles from the different isolation steps are compared, it is evident that also substances with molecular weights below the nominal cut-off of the membranes used are present as contaminants in higher-molecular-weight fractions. These solutes sometimes become detectable only after enrichment during the preparative steps, *i.e.*, their concentrations before are below the analytical detection threshold. The importance of this finding for the interpretation of bioassays is evident from the results in Table I. Subfractions from uraemic haemofiltrates after reverse osmosis, ion-exchange chromatography and finally size exclusion chromatography were added to rat bone marrow cells *in vitro* and the [^{3}H]thymidine incorporation into the cells was measured. Additionally, the peaks were analyzed by thin-layer chromatography (butanol-1-acetic acid-water, 4:1:1) and stained with ninhydrin or Pauly reagent (diazotized sulphanilic acid) (see Table I).

The toxicity of the subfractions described, although not homogeneous as yet, can be summarized: toxic fractions differ as well in their molecular weights as in their

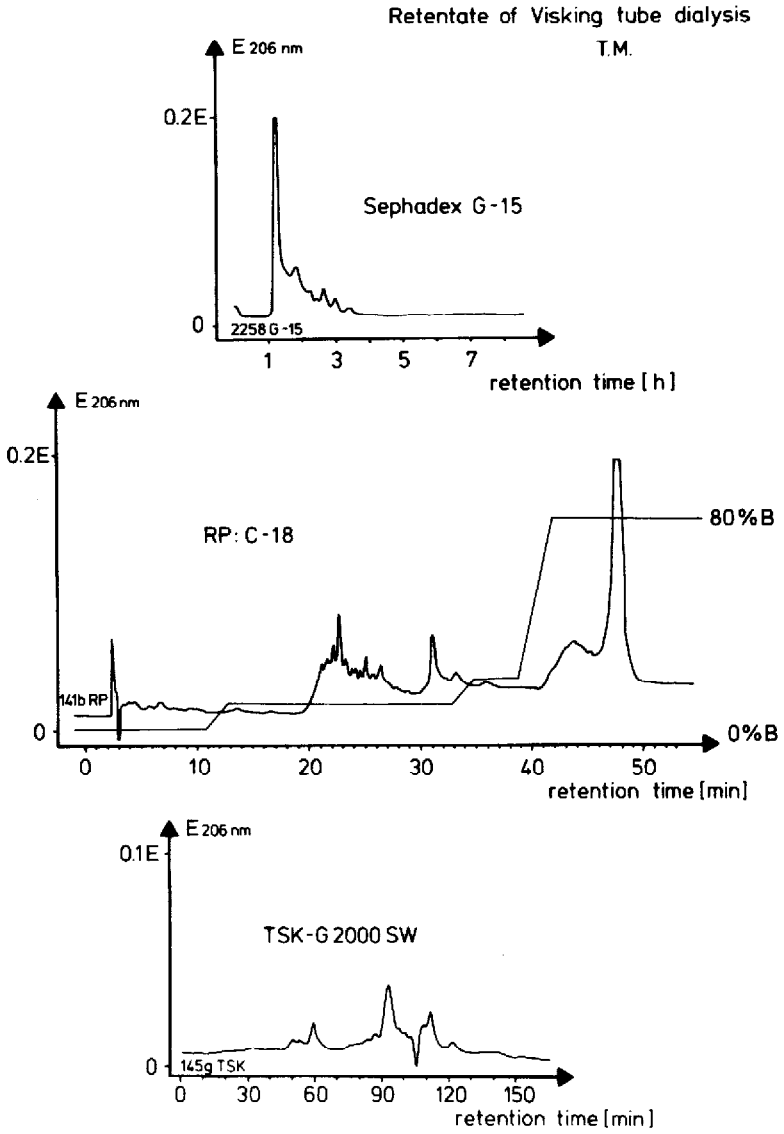

Fig. 5. Heterogeneity of the high-molecular-weight retentate isolated from haemofiltrate using dialysis in Visking tubes *in vitro* as shown by conventional size exclusion chromatography (upper part, conditions as in Fig. 3), high-performance size exclusion chromatography (lower part, conditions as in Fig. 1) and reversed-phase chromatography (middle part, conditions as in Fig. 2).

net charges at pH 8.6. As expected, several acidic fractions inhibit [^{3}H]thymidine incorporation into rat bone marrow cells *in vitro*. These contain peptides. In addition, subpeaks from fraction A, which is eluted under the starting conditions from the anion exchanger, significantly inhibit DNA synthesis of the cells. These fractions show ninhydrin and Pauly reagent-positive spots after thin-layer chromatography. These findings agree with the results of Dzúrik *et al.*[3], Menyhárt and Gróf[16], Lutz[17],

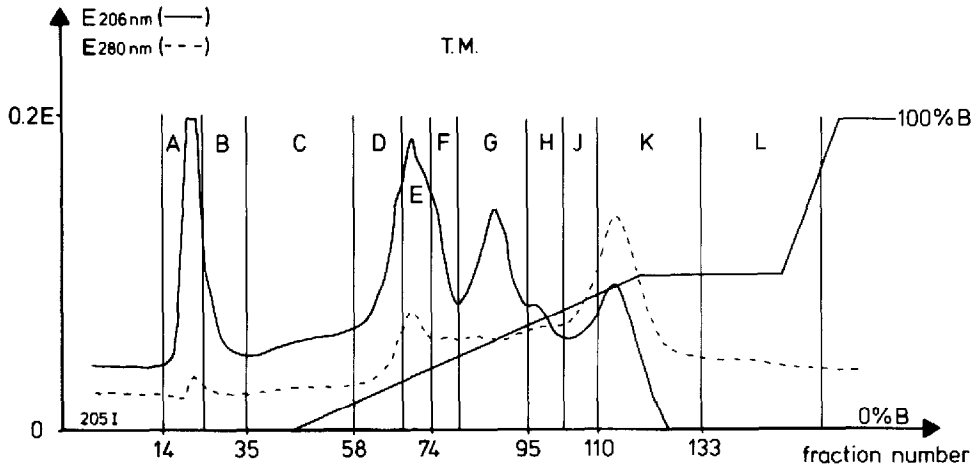

Fig. 6. Preparative anion-exchange chromatography of 1 g desalted and concentrated haemofiltrate (DEAE-Sephacel; double column technique, each 23 × 5 cm; 5°C; 300 drops per fraction, linear flow-rate 5.15 cm/h).

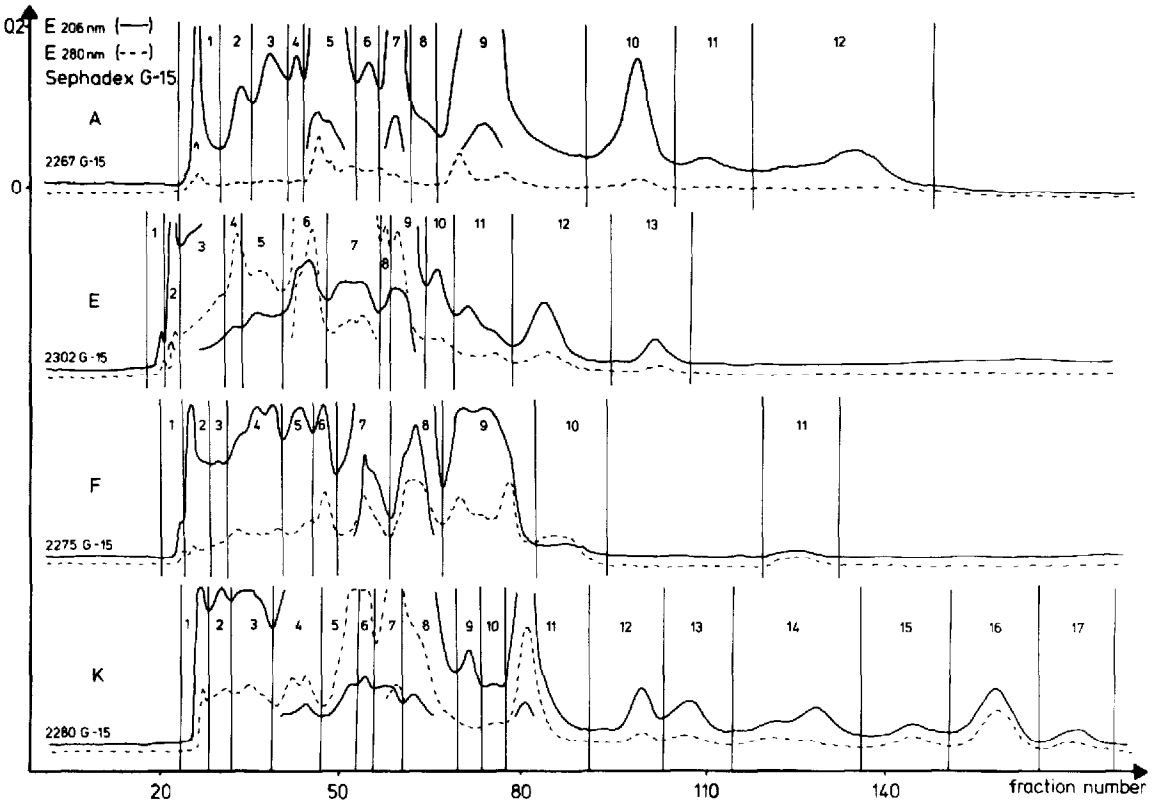

Fig. 7. Preparative rechromatography of peaks A (2946 mg), E (799 mg), F (825 mg), and K (498 mg), respectively, from ion-exchange chromatography (see Fig. 6) on Sephadex G-15. Column: 100 × 5 cm. Eluent: 0.05 mol/l NH_4HCO_3; linear flow-rate 3.3 cm/h. Temperature: 5°C.

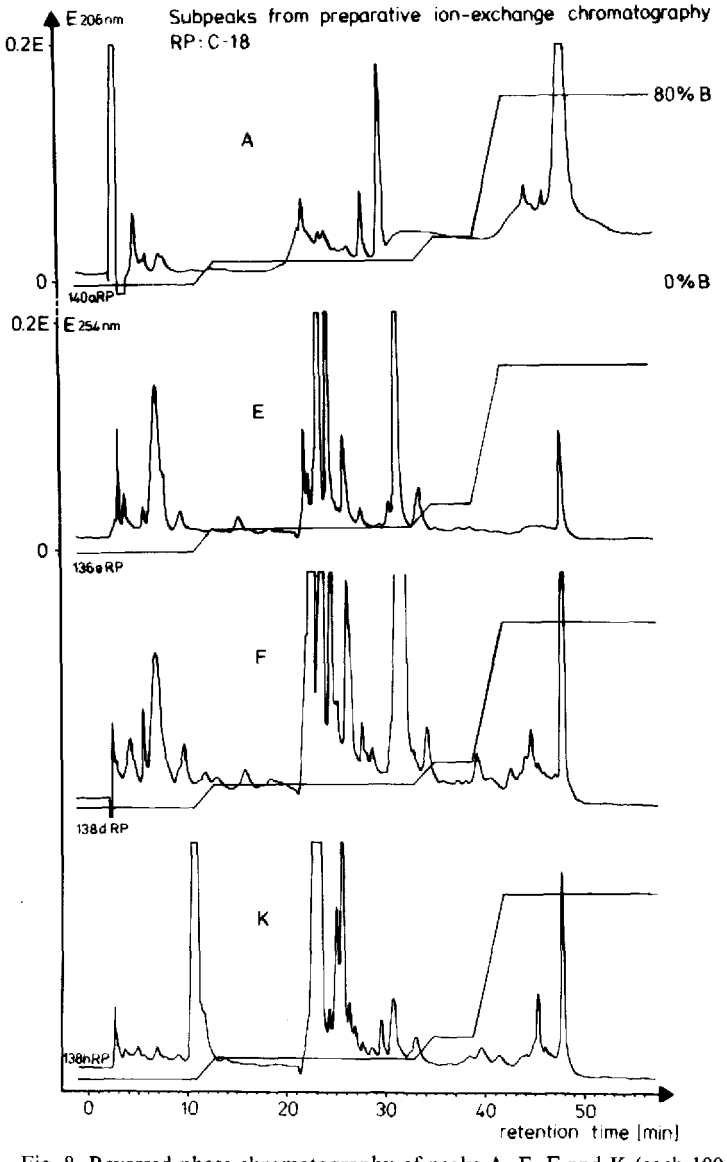

Fig. 8. Reversed-phase chromatography of peaks A, E, F and K (each 100 μl) from ion-exchange chromatography. Conditions as in Fig. 2.

Kinniburgh and Boyd[18], Traeger *et al.*[19] and Contreras *et al.*[20]. On the other hand, the extreme heterogeneity is in good accord with the results of Mabuchi and Nakahashi[21] and Schoots *et al.*[22].

CONCLUSIONS

The described combination of membrane separation techniques and conventional and high-performance liquid chromatography enables the preparative isolation

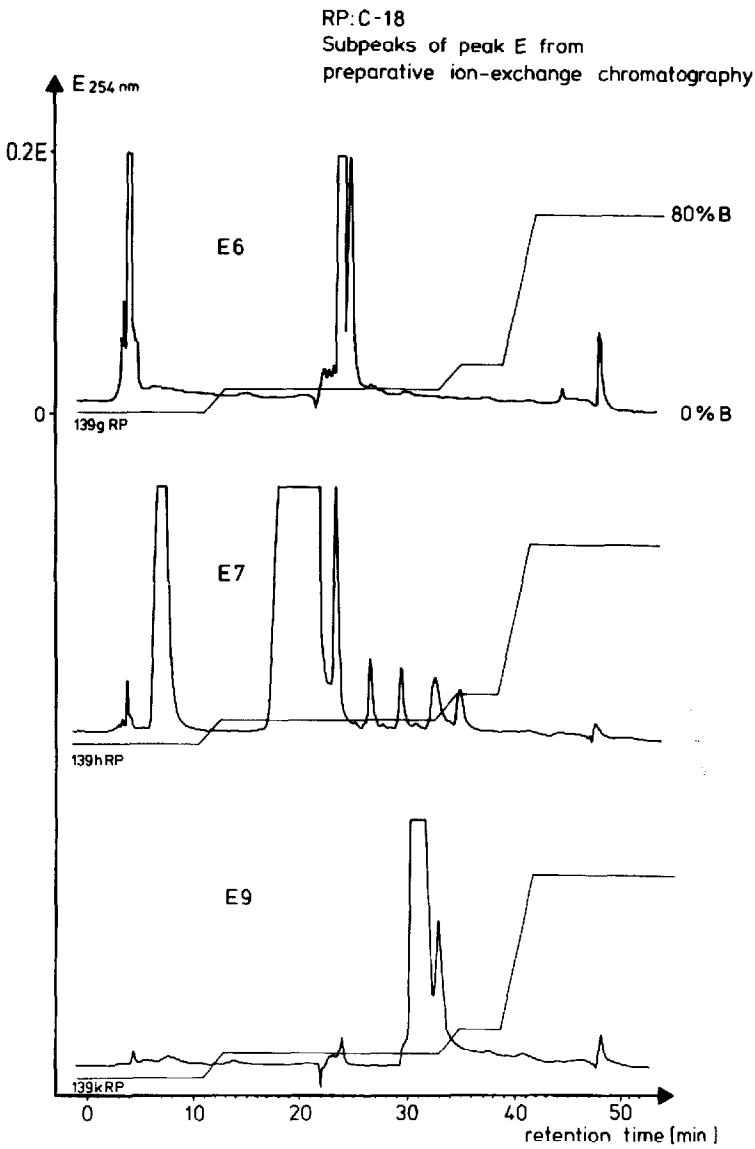

Fig. 9. Rechromatography of 100 μl of peaks E6, E7 and E9, respectively, from preparative gel chromatography (see Fig. 7) using reversed-phase chromatography, conditions as in Fig. 2.

and analytical investigation of a broad range of biologically active subfractions from extremely heterogeneous and diluted biological fluids. Using fluids from the artificial kidney, middle- and higher-molecular-weight fractions from uraemic sera become available. The molecular weight distribution of fractions, which inhibit DNA synthesis *in vitro*, far exceeds those usually found: the distribution of net charges includes basic, neutral and acidic species. Several contain peptides. Whether these are responsible for the inhibitory activities remains, as yet, unclear. Also, the question of

TABLE I

[^{3}H]THYMIDINE INCORPORATION INTO RAT BONE MARROW CELLS *IN VITRO* AFTER ADDITION OF SUBFRACTIONS ISOLATED FROM HAEMOFILTRATE USING REVERSE OSMOSIS, ION-EXCHANGE CHROMATOGRAPHY AND SIZE EXCLUSION CHROMATOGRAPHY (SEE FIG. 7)

Values are given as per cent incorporation relative to cultures without any test fraction, as means and standard deviations from five cultures (500 μg fraction added to 5 × 10^5 cells). The two right-hand columns show the staining behaviour of subfractions after thin-layer chromatography (n.d. = not determined)

Fraction	*[^{3}H]thymidine incorporation*	*No. of ninhydrin-positive spots*	*No. of Pauly reagent-positive spots*
A 1	54.9 ± 2.0	n.d.	n.d.
A 2	27.2 ± 0.7	8	1
A 3	33.2 ± 2.4	5	0
A 4	30.7 ± 0.7	7	1
A 5	36.3 ± 5.2	6	1
A 6	60.3 ± 4.8	2	1
A 7	41.5 ± 2.0	2	2
A 8	3.5 ± 1.5	3	3
A 9	8.2 ± 4.0	2	0
A 10	5.1 ± 1.5	2	0
A 11	5.0 ± 0.5	3	2
A 12	8.5 ± 1.7	2	0
F 1	14.7 ± 0.6	n.d.	n.d.
F 2	23.0 ± 1.3	5	0
F 3	49.5 ± 5.4	5	0
F 4	32.4 ± 3.9	3	2
F 5	35.6 ± 2.2	7	1
F 6	n.d.	7	1
F 7	19.0 ± 1.0	2	2
F 8	16.5 ± 0.6	3	2
F 9	7.5 ± 0.6	4	5
F 10	14.4 ± 0.9	4	0
F 11	12.1 ± 5.0	3	0

clinical relevance cannot be answered by these experiments. However, all the results show that the heterogeneity of "uraemic toxins", especially in the middle- and higher-molecular-weight region, may be higher than expected.

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