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ANALYTICAL ISOTACHOPHORESIS OF UREMIC BLOOD SAMPLES

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SUMMARY

Uremic blood samples were analyzed for ionogenic substances using analytical isotachophoresis. Multicomponent separations proved that the uremic state shows significant differences from the normal state, especially with regard to anionic low-molecular-weight substances. As a quantitative parameter the ratio of anionic higher-molecular-weight substances to anionic low-molecular-weight substances is proposed: the HL ratio. Separation patterns and HL ratios were studied during nine weeks for one patient on chronic hemodialysis. The patient showed a low HL ratio due to excess of low-molecular-weight substances. Separation patterns before and after hemodialysis showed clear differences and the HL ratio increased. The method of analysis is neither time- nor sample-consuming and sample preparation is not needed. Experimental procedures are easily standardized and results are reliable.

INTRODUCTION

Despite the fact that in recent years considerable progress has been made in hemodialysis techniques, patients still suffer from substantial disabilities. For a long time symptoms of uremic intoxication were attributed to excess of low-molecular-weight substances [1]. The possible importance of middle-molecular-weight substances was established when Babb et al. [2] suggested the middle molecule hypothesis.

Obviously the uremic state is in many respects different from the normal state, not least of which is the biochemical aspect [3]. The clinical state of patients suffering from renal failure can be improved by dialysis of the blood, thus removing the toxic products of metabolism that would normally be excreted by the kidneys. However, solutes removed by the artificial "kidney" may differ from those eliminated by a normally functioning kidney. Moreover some are eliminated in excess and others are poorly removed. As a result, concentrations of many metabolites in the body fluids of uremic patients are different from those of normal subjects.

The search for uremic toxins has been highly specific, assessing the possible toxicity of single known substances. Due to the complex and nonspecific nature of uremic toxins and their interrelations, such studies are rather elaborate and difficult. In recent years, however, some research groups have followed a screening approach to the problem [4, 5], in which chemical multicomponent analyses of biological fluids, derived from uremic patients, play an important part. The obvious drawback of such an approach is the extraordinary complexity of such fluids.

Analytical chemistry has been successful in the development of techniques that permit separation, identification, quantification and even isolation of many metabolites in biological fluids. For the qualitative and quantitative evaluation of ionic solutes several analytical techniques are available and each has its own disadvantages and limitations. Isotachophoresis [6] is a rather new analytical technique that seems to be compatible with the basic requirements for screening approach procedures: multicomponent information, rapid completion, reliable and inexpensive. Moreover the flexibility of the technique offers a vast spectrum of possibilities for detailed studies. Hence isotachophoresis can provide useful information on the occurrence of ionic solutes in uremic biological fluids.

EXPERIMENTAL

All isotachophoretic experiments were performed in the equipment developed by Everaerts and coworkers [6—8]. The separation capillary was 20 cm × 0.2 mm I.D. Samples were introduced by means of a microliter syringe. The electrical driving current was stabilized at 20 μA. Voltages varied between 1 and 16 kV. Separation times were less than 20 min. Separated zones were detected by means of conductimetric and UV (254 nm) detection systems.

All chemicals used were of analytical grade or additionally purified by conventional methods. Water was of double distilled quality. Exact specifications of the electrolyte systems are given in Tables I and II. In both systems anionic solutes can be analyzed. The operational system of Table I has a large difference between the mobilities of the leading and the terminating ionic species. Due to this large mobility gap the system is particularly suitable for screening approach procedures. As a more differentiating system the operational system of Table II was chosen.

Blood samples were obtained from a 61-year-old female patient with polycystic renal disease, on intermittent hemodialysis since August 6th, 1973.

TABLE I

OPERATIONAL SYSTEM FOR THE DETERMINATION OF THE HL RATIO

	Electrolyte	
	Leading	Terminating
Anion	Chloride	HEPES*
Concentration	0.01 *M*	0.01 *M*
Counterion	Histidine	TRIS**
pH	6.02	8.50
Additive	0.1% HEC***	
Solvent	Water	
Current density	0.064 A/cm^2	
Temperature (°C)	Ambient air, 21°	

*HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma, St. Louis, Mo., U.S.A.) recristallized from water—ethanol.
**TRIS = Tris(hydroxymethyl)aminomethane (Merck, Darmstadt, G.F.R.).
***HEC = hydroxyethylcellulose (Cat. No. 5568 Polysciences, Warrington, Pa., U.S.A.); a 1% stock solution was purified by ion exchange (Ionenaustauscher V; Merck).

TABLE II

OPERATIONAL SYSTEM FOR ANIONIC SEPARATION AT LOW pH

	Electrolyte	
	Leading	Terminating
Anion	Chloride	Caproate
Concentration	0.01 *M*	0.01 *M*
Counterion	β-Alanine	Na^+
pH	3.75	6.20
Additive	0.1% HEC*	
Solvent	Water	
Current density	0.064 A/cm^2	
Temperature (°C)	Ambient air, 21°	

*HEC = hydroxyethylcellulose.

Dialysis was performed with a poly-acrylonitrile membrane three times per week for 4 h in a RP6 open system [9]. The dialysate composition is given in Table III. During a period of 2½ months, 1.5-ml blood samples were taken in EDTA just before and immediately after hemodialysis. After centrifugation, samples were stored at −20°. Before analysis, samples were diluted eleven fold with double-distilled water. Reference samples were obtained from several healthy subjects and analyzed under the same experimental conditions.

RESULTS

In applying isotachophoresis as a screening approach much information must be obtained in a relatively short analysis time. We therefore limited the time of

TABLE III

COMPOSITION OF THE DIALYSATE

Sodium	Na^+	134.5 mequiv./l
Potassium	K^+	1.0 mequiv./l
Acetate	CH_3COO^-	45.0 mequiv./l
Chloride	Cl^-	95.5 mequiv./l
Magnesium	Mg^{++}	1.0 mequiv./l
Calcium	Ca^{++}	4.0 mequiv./l

analysis to 20 min at moderate current densities. Concerning the multicomponent information, a methodological choice has to be made between anionic and cationic separations. Preliminary experiments showed that anionic separations give more information, so we confined our investigations to the anodic separation mode. Fig. 1 gives a representative wide range result of a predialysis uremic blood sample, analyzed in the operational system of Table I. From the linear conductivity tracing (Fig. 1 LIN), it can be seen that there are many anionic solutes present within this sample, as indicated by the stepwise change of the conductivity signal.

Qualitative information, for identification purposes, is obtained in isotachophoresis from the conductance of a zone relative to that of the leading zone [6, 8]. The reciprocal conductance axis, at the left hand side of Fig. 1, has been calibrated with respect to this. It should be emphasized that within a

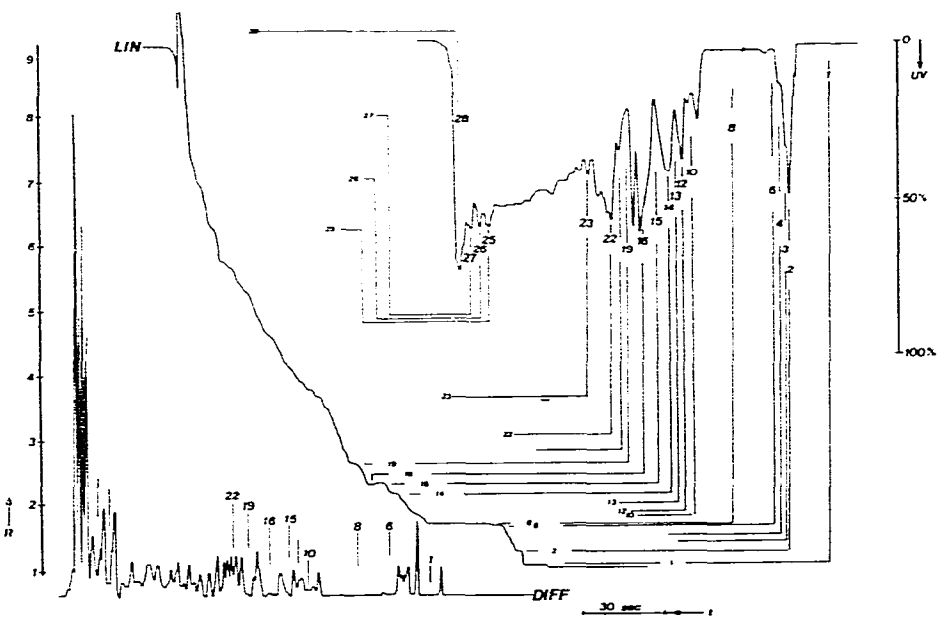

Fig. 1. Uremic plasma sample analyzed in the operational system of Table I. Sample: elevenfold diluted plasma from J.B. (3 μl). 0 = Chloride, 1 = sulphate, 5 = acetate, 6 = lactate, 8 = phosphate, 14 = hippurate, 16 = urate. R = resistance; t = time; UV = UV absorption 254 nm. For further explanation see text.

given operational system relative stepheights are species specific. Hence component number 8 (phosphate) will always migrate at a relative stepheight of $R = 1.70$, whatever the nature of the sample.

Additional information is obtained when the sample zones are migrating through the UV detection system. Each zone will have or not have a specific UV absorption at 254 nm. Since in isotachophoresis the steady-state configuration is no longer a function of time, the UV tracing (Fig. 1 UV) can be superimposed upon the conductivity tracing. In this way, zones that contain UV absorbing solutes are easily localized. For a number of zones the relationship between the UV and the conductivity tracing is indicated by numbered lines. Some solutes are given in the legend of Fig. 1. The advantage of the dual detection system is obvious from the fact that a non-UV-absorbing solute like sulphate, cannot be located by the UV detector. Its presence is nevertheless clearly indicated by the conductometric detection system (Fig. 1, LIN No. 1). Due to the large mobility gap and the large number of solutes that are present, several mixed zones, stable with respect to time, can be expected. The numbering of the zones therefore is based upon the characteristics of the UV profile.

In isotachophoresis the length of a zone is proportional to the amount of the ionic species in the sample. In order to facilitate the measurement of zone lengths the linear conductivity signal has been differentiated. From the differential tracing (Fig. 1, DIFF) distances between zone boundaries can be measured. Sample concentrations are calculated from the measured zone length by methods of calibration or using an internal standard [6, 8].

Several uremic patients have been screened within this operational system and they all show similar profiles, though minor individual differences occur. In order to assess the difference between the normal and the uremic state, blood samples of normal subjects were analyzed. Representative UV separation profiles of four normal subjects are given in Fig. 2. Differences with the uremic state are obvious, especially in the low-molecular-weight region. This region comprises the zones 1 up to 22 and has a relative conductance $G_R < 3$. Fig. 3 shows a blank run of the operational system of Table I and analyses of samples derived from a uremic patient before and after hemodialysis. From Fig. 3a it can be seen that there is only a small impurity present originating from the electrolyte system. Though the separation profiles of Figs. 3b and 3c show a large similarity, it can be concluded from the decrease in UV absorption that the sample after hemodialysis, Fig. 3c, contains fewer UV absorbing solutes. Moreover in Fig. 3c, acetate, originating from the dialysate is clearly visible. In the legend of Fig. 1 some other solutes are given.

Since the excess of lower-molecular-weight substances is a characteristic difference between the normal and the uremic state it is advantageous to describe this difference as a single quantitative parameter. The ratio of anionic higher-molecular-weight substances to anionic low-molecular-weight substances seems appropriate, in which the HL ratio is given by the quotient of two zone-lengths:

$$HL = (\Delta l_{28-22}) : (\Delta l_{22-2})$$

Most anionic low-molecular-weight substances, mol. wt. < 400, have low dis-

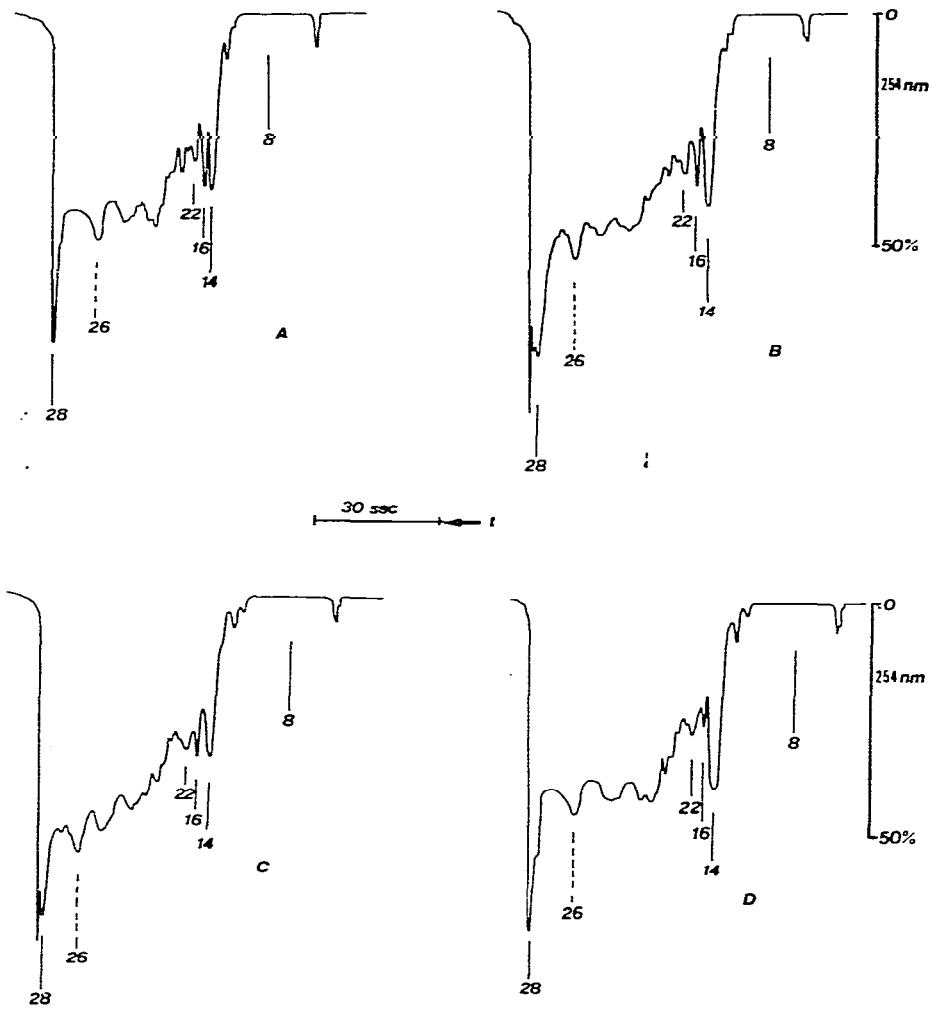

Fig. 2. Normal plasma samples analyzed in the operational system of Table I. Samples: eleven-fold diluted plasma (3 μl). A: female, B: male, C: female, D: male. UV (254 nm) tracings; t = time.

sociation constants, $pK_a < 6$, and migrate within the operational system of Table I as rather mobile species. They will generally migrate at a low relative conductance, i.e. $G_R < 3$. Higher-molecular-weight substances like peptides have higher dissociation constants and a low ionic mobility and for that reason migrate in the less mobile part, $G_R < 3$. Most amino acids have fairly high pK_a values and will not migrate isotachophoretically within this operational system. It should be noticed that even relatively high-molecular-weight substances, like heparin, can migrate as rather mobile species, provided they have a large net electrical charge. As a criterion for finding the ratio we chose zone No. 22, since this zone seems characteristic for all separations done up until now. In order to study the relevancy of this ratio a patient was screened during several weeks of hemodialysis. Samples were analyzed according to the operational

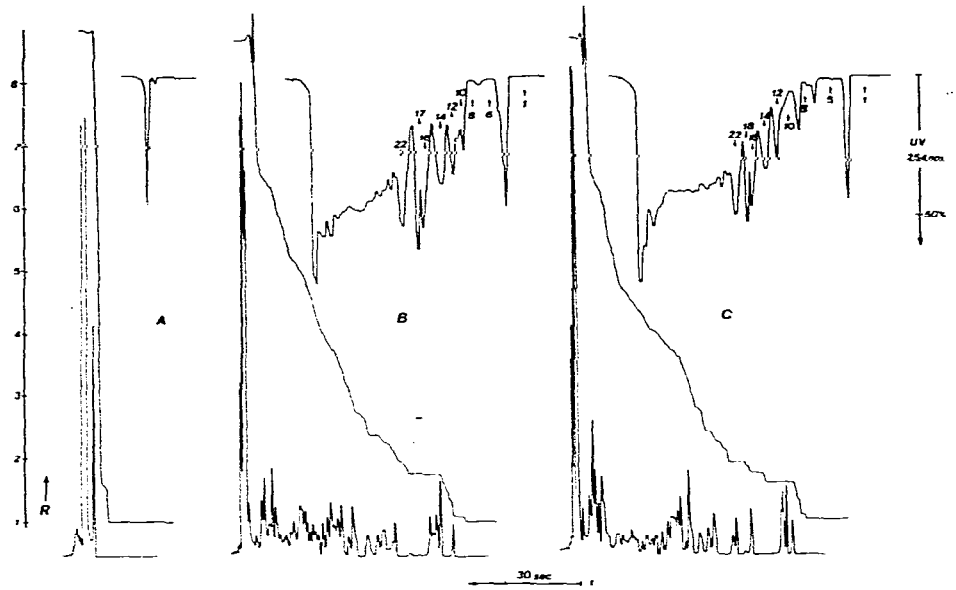

Fig. 3. Uremic plasma samples analyzed in the operational system of Table I. Sample: eleven-fold diluted plasma from T.M. (3 μl). A: Blank run, B: predialysis, C: postdialysis.

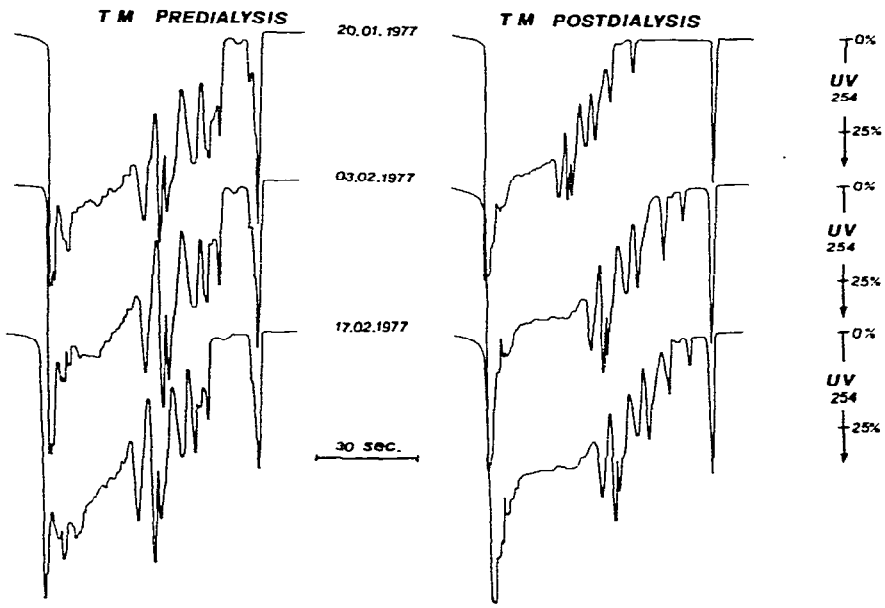

Fig. 4. The longitudinal behaviour of sample from patient T.M. Operational system: Table I. Sample: eleven-fold diluted plasma (3 μl).

system of Table I. Some representative results are given in Fig. 4. Comparison of these results with the UV tracings from Figs. 1 and 3 indicates the strong similarity of the separation patterns, especially in the lower-molecular-weight region. Moreover the strong resemblance of the separation profiles for different data suggests a fairly constant response from the patient to intermittent

hemodialysis. In Table IV the measured HL ratios have been summarized. Normal values are significantly higher than unity, whereas predialysis values are smaller than unity due to the excess of anionic low-molecular-weight substances. Post-dialysis values, corrected for acetate, are slightly larger than unity.

TABLE IV
HL RATIOS: NORMAL-, PRE- AND POSTDIALYSIS VALUES

										Mean	C.V. (%)
Normal	1.10	1.25	1.22	1.17							
T.M. pre	0.86	0.91	0.91	0.79	0.86	0.84	0.77	0.79	0.72	0.83	8
T.M. post	0.93	0.79	0.81	0.46	0.84	0.80	1.00	0.77	0.77	0.80	20
T.M. post*	1.19	1.15	0.94	0.90	1.10	1.03	1.21	0.98	1.00	1.06	10

*Corrected for acetate content.

With the operational system of Table II blood samples were analyzed for the content of weakly acidic and acidic anionic solutes of low molecular weight. Fig. 5a shows a representative result of a normal serum in which phosphate and lactate can be determined. Fig. 5 b and c shows the same samples as Fig. 3 but now analyzed within the operational system of Table II. The differentiating capabilities of this system are better compared to those of Table I, but the mobility gap is considerably smaller. For the determination of concentrations this system is more appropriate. From a calibration run the concentrations of three constituents could be determined. Results are given in Table V.

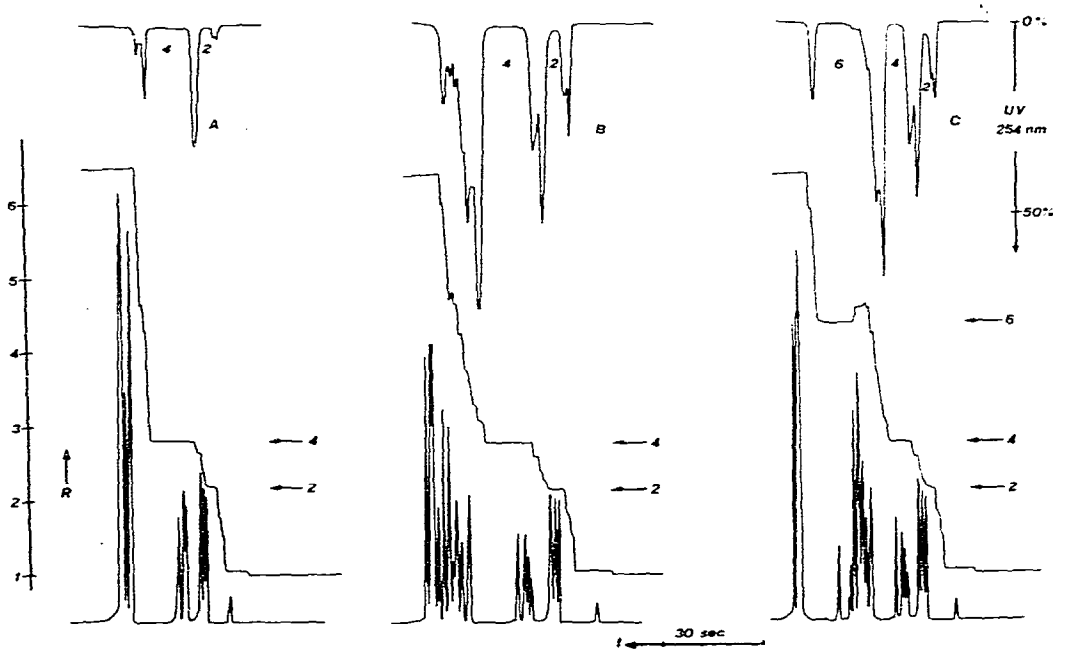

Fig. 5. Normal and uremic samples analyzed in the operational system of Table II. Samples: eleven-fold diluted (3 μl). A: Normal, B: T.M. predialysis, C: T.M. postdialysis. 2 = Phosphate; 4 = lactate; 6 = acetate.

TABLE V

DETERMINED CONCENTRATIONS (m*M*)

	Normal	T.M. predialysis	T.M. postdialysis
Phosphate	0.9	1.8	1.1
Lactate	3.9	4.8	2.5
Acetate	Trace	Trace	4.4

DISCUSSION

Isotachophoresis provides multicomponent information of uremic blood samples in a short analysis time. Due to the minimal sample preparation and the rigidly standardized experimental conditions results have good reproducibility and a high reliability.

For the screening approach of anionic solutes in uremic blood samples we used a wide range electrolyte system, for which the total time of analysis is less than 20 min. The fairly high dissociation constant of the terminating ion N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, $pK_a = 7.55$, guarantees that almost all acidic and weakly acidic solutes are being analyzed. The majority will migrate isotachophoretically as rather mobile anions: Fig. 1, $G_R < 3$. Moreover various peptides and peptide-like substances will be analyzed without rigid limitation to molecular weight: Fig. 1, $G_R > 3$. The resolving capabilities of this electrolyte system will be relatively poor for the less mobile part of the mobility gap. In the mobile part, $G_R < 3$, acidic and weakly acidic organic and inorganic solutes are separated with satisfactory differentiation.

Bultitude and Newham [5] have reported a method for comparing plasma samples from patients with chronic uremia before and after dialysis, using combined gas chromatography—mass spectrometry. They showed that the concentrations of several low-molecular-weight substances increase in uremia. Comparison of the normal sera separations of Fig. 2 with the uremic sample separations, Figs. 1, 3 and 4, confirm these findings. After dialysis Bultitude found that concentrations became approximately the same as from plasma of healthy subjects. Considering the low-molecular-weight organic acids we must conclude that, though individual concentrations are lower after hemodialysis, they are still considerably higher than normal values. This means that the concentration level of several of these substances will be in the millimolar range. Identification and quantification of more solutes is the subject of present investigations.

During hemodialysis only a slight change in the higher-molecular-weight region has occurred. This region seems of particular interest since the growth of evidence showing the importance of substances of middle molecular weight: mol. wt. 400—5000 [2, 4, 10, 11]. Most analytical information about these substances has been obtained with gel chromatography [4, 10, 11]. Although little is known of the exact nature of these substances, undoubtedly some will be analyzed within the wide range system of Table I. Gel chromatographic studies have proved the removal of middle molecules by adequate hemodialysis

[4, 10]. Our isotachophoretic experiments suggest only a small change in the higher-molecular-weight region. It should be emphasized however, that the separation mechanism of isotachophoresis is quite different from that of gel chromatography. Hence the higher-molecular-weight substances, as analyzed by isotachophoresis, will be different from those separated by gel chromatography.

Considering the HL ratio it can be concluded that the uremic state is characterized by a relatively low ratio, due to excess of organic acids. From Table V it follows that the ratio after hemodialysis has not changed significantly, due to the high acetate concentration originating from the dialysate. Moreover variations in the acetate content are responsible for the high value of the coefficient of variation. Since acetate is assumed to be metabolized rather quickly [12], it seems appropriate to correct the HL ratio for the acetate content. The increase of the ratio after hemodialysis then suggests a slight correction of the uremic state. This correction is also demonstrated by the more differentiated separations in the operational system of Table II. Within this operational system the metabolically important constituent acetate [13] can be easily determined. Further work on the determination of HL ratios in different hemodialysis strategies, identification and quantification of uremic solutes is the subject of current investigations.

We conclude that isotachophoresis is compatible with most of the demands for screening approach procedures. Particularly in the field of ionic solutes, isotachophoresis proves to be a useful analytical tool.

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