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ION-EXCHANGE DERIVATIVES OF SPHERON

II. DIETHYLAMINOETHYL SPHERON*

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SUMMARY

The preparation of diethylaminoethyl derivatives of Spheron (DEAE-Spheron 300) having different capacities for small ions (nominal capacities 0.11, 0.26, 0.60, 1,23, 1.63, 2.05 and 2.20 mequiv./g) is described. Routes leading to a high degree of ionogenic substitution are discussed. The ion exchangers were characterized analytically and by titration curves, which indicate that the ion exchangers are homoionic. The chromatographic properties of the DEAE-Spherons were tested in the chromatography of serum albumin, of an artificial mixture of proteins (lysozyme, chymotrypsinogen and serum albumin), blood plasma, a mixture of peptides and of glutamic, aspartic and cysteic acids using an amino acid analyzer and also in the chromatography of adenosine phosphates. In all cases the best separations were obtained with anion exchangers having the highest nominal capacities. The effect of degree of ionogenic substitution on the decrease of the partial hydrophobicity of the Spheron matrix is discussed. It is shown that at a nominal capacity exceeding 1.2 mequiv./g the effect of hydrophilic iogenic groups balances the partial hydrophobicity of the Spheron matrix, so that no hydrophobic sorption of proteins takes place.

INTRODUCTION

Until the mid-1960s, the development of ion-exchange chromatography of biopolymers was retarded by the lack of suitable hydrophilic ion exchangers. The strongly hydrophobic ion-exchange resins available, possessing an aromatic matrix, often denatured the biopolymers. The more hydrophilic carboxylic cation exchangers having a polyacrylic matrix sorbed the biopolymers on their surface only, owing to

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their low average pore size. Chromatographic methods could be successfully applied to the analysis of sensitive macromolecular biopolymers only after hydrophilic cellulose had been introduced by Peterson and Sober[1–3] as a fibrous matrix for ion exchange. These possibilities were extended by Porath and Lindner[4] who introduced the polydextran matrix for ionogenic substitution. Cellulose and polydextran ion exchangers contributed considerably to the development of modern biochemistry, but could not be utilized in the high-performance liquid chromatography (HPLC) of biopolymers.

At present, hydrophilic ion-exchange derivatives with a matrix made of desulphurized and crosslinked agarose[5–7] and of porous glass coated with a covalently bound hydrophilic glycerol monolayer[8–11] are also available for chromatographic purposes. All the hydrophilic matrices mentioned so far were ionogenically substituted with diethylaminoethyl (DEAE) or carboxymethyl functional groups; some of them were also substituted with the sulphopropyl or quaternary aminoethyl group.

We have shown[12,13] that hydrophilic macroporous ion-exchange derivatives suitable for the chromatography of biopolymers and their fragments may be prepared by ionogenic substitution of the hydroxyalkyl methacrylate matrix of the Spheron™ gel. These macroreticular ion-exchange resins exhibit sufficient capacity and very good flow parameters in column chromatography and are promising for the application of HPLC to the separation of biopolymers. Part I of this series[13] contained a more detailed characterization of this gel and the description of its preliminary treatment, allowing adequate ion exchange resins to be obtained by ionogenic substitution. This paper reports the preparation and characterization of DEAE-Spheron, which is the most promising material with respect to chromatographic applications in biochemistry and similar fields.

EXPERIMENTAL

Materials

Spheron P-300, particle size 20–40 μm, was an extracted sample, the preparation (from products manufactured by Lachema, Brno, Czechoslovakia) and characterization of which have been described in Part I[13]. An analogous microparticular (10 μm) carrier is manufactured by the Laboratory Instruments Works (Prague, Czechoslovakia) under the trade name of Separon HEMA-300. N-2-Chloroethyl-N, N-diethylammonium hydrochloride was a product of E. Merck (Darmstadt, G.F.R.). Reagent grade chemicals and sugars were supplied by Lachema. The individual proteins (serum albumin, chymotrypsinogen, lysozyme) were of the same origin as in Part I[13]. Lyophilized human blood plasma was supplied by the Institute of Sera and Vaccines, Prague, Czechoslovakia. The partial pancreatin hydrolyzate of casein was a Trypton product of Oxoid, London, Great Britain. Amino acids were standards for analysis, produced by the National Biochemical Corp., Cleveland, Ohio, U.S.A. L-Cysteic acid was a product of Calbiochem, Luzern, Switzerland. Adenosine phosphates were identical with samples described in ref. 12.

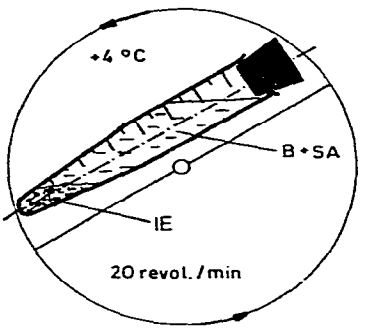
Methods

DEAE-Spheron 300 was prepared (*cf.*, ref. 14) from Spheron P-300, preextracted according to ref. 13, which was stirred in a solution of potassium hydroxide,

by adding N-2-chloroethyl-N, N-diethylammonium hydrochloride after swelling. The reaction was completed at higher temperature. Larger capacities were achieved by repeated reaction. The product was decanted several times from water until there was no turbidity in the supernatant, then several times from dilute hydrochloric acid and sodium hydroxide. Finally, the product was washed with methanol and acetone and dried *in vacuo* at 20°/15 mmHg for 3 days.

The DEAE-Spheron thus obtained was characterized (determination of the dry matter, elemental analysis, swelling, determination of the working volume, exclusion limit, inner surface, nonpenetratable volume and capacity for small ions) as described in detail in Part I[13]. The titration curves were determined by using Titrator TTT 1a and Titrigraph SBR2c (Radiometer, Copenhagen, Denmark).

The capacity for proteins was determined by the static method using conic test-tubes, slowly rotating in a refrigerator at 4° (*cf.*, Fig. 1). These test-tubes were also used for the regeneration, cycling and equilibration of the ion exchange resin before the determination of capacities. A 250-mg amount of the Spheron ion-exchange derivative in a test-tube was covered with distilled water, stirred, deaerated and centrifuged and treated three times by the following procedure: stirring with the regenerant (*ca.* 5–8 ml); centrifuging after 5 min; removal of the regenerant by suction through a capillary to a height of 0.5 cm over the sedimented ion-exchange layer,



Ion exchanger (IE)	0·25 g
IE + buffer (B)	5·00 ml
6 % sol. of serum albumin (SA)	5·00 ml
Sorption	16 h
Desorption	4 h

Fig. 1. Device for the determination of the capacity of Spheron ion-exchange resins for proteins by static method. Conical test-tubes provided with closure, *ca.* 12–15 ml volume and calibrated to 10.0 ± 0.1 ml, were fixed on a plate which slowly rotated in a refrigerator. Four working test-tubes and one blank could be fixed simultaneously on one plate. The working test-tubes contained a weighed amount of the ion-exchange resin and protein solution of known concentration in a sorption buffer having low ionic strength. After the establishment of equilibrium and centrifugation, the loss in protein concentration in 5.0 ml of supernatant was determined spectrophotometrically. Desorption was effected by substituting the volume of solution taken from the tube by 5 ml of buffer having high ionic strength, followed by repeated slow stirring. After the establishment of equilibrium, the increase in protein concentration was determined spectrophotometrically (see text).

which must not be stirred. The regenerant used, was always 2 *M* sodium chloride and water followed, for the anion-exchange resins, successively by 2 *M* hydrochloric acid, water, 2 *M* sodium hydroxide and water, and the resin remained in the OH^- form. (For the cation-exchange resins, the order employed was: 2 *M* sodium hydroxide, water, 2 *M* hydrochloric acid and water; the resin remained in the H^+ form.) The ion-exchange resins were equilibrated by repeated stirring with the sorption buffer solution, in a similar way to the regenerants, until the supernatant removed by suction had

the same pH, conductivity and absorbancy values as the sorption buffer solution added. The sorption buffer solution for capacity measurements of serum albumin was 0.01 *M* sodium acetate, pH 6.5.

To the equilibrated ion-exchange resin, from which the final supernatant had been removed by suction, were added 5.00 ml of the protein stock solution (3.00 g of serum albumin dissolved in the sorption buffer solution in a 50 ml flask) and the solution was made up to 10 ml with the sorption buffer. Another volume of the sorption buffer solution was added, equal to one quarter of the non-penetrable volume ($n/4$ ml, usually 0.20 ml; *cf.*, ref. 13 and Table I). At the same time, 5.00 ml of the same protein stock solution and 5.00 ml of the sorption buffer were added into a blank test-tube. Both test-tubes were then closed and left to rotate slowly for 16 h (overnight). The contents of the two test-tubes were centrifuged, and 5.00 ml of solution were taken from each, placed in 50 ml flasks and made up to the mark with 45 ml of the sorption buffer. The protein content in these dilute solutions was determined by UV absorbancy measurements at 280 nm. If the equilibrium desorption of proteins was to be determined, 5.00 ml of the desorption buffer solution were added to each of the two test-tubes. (The desorption buffer solution consisted of sorption buffer solution to which sodium chloride had been added to give a concentration of 2 *M*.) The test-tubes were left to rotate for another 4 h, centrifuged, then 5.00 ml from each were transferred into a 25 ml flask. A 20-ml volume of the desorption buffer diluted 1:1 with the sorption buffer was added to each flask, and the protein concentration was determined photometrically. The results from the measurements were obtained as follows.

The protein contents (in mg/10 ml) are designated: after sorption in the working test-tube, W_s; in the blank test-tube, B_s; after desorption in the working test-tube, W_d; in the blank test-tube, B_d. The equilibrium sorption S in milligrams of protein per gram of dry Spheron is

$$S = 4(B_s - W_s) \tag{1}$$

and the equilibrium desorption D expressed in the same units is

$$D = 4\left(W_d - \frac{W_s}{2}\right) \tag{2}$$

B_d was used only for checking.

The preparation of DEAE-Spheron for chromatographic tests (swelling, deaeration, regeneration with 2 *M* sodium chloride, cycling with 2 *M* hydrochloric acid, water, 2 *M* sodium hydroxide and water and equilibration with buffers) was carried out on a fritted filter by employing described procedures[12,13].

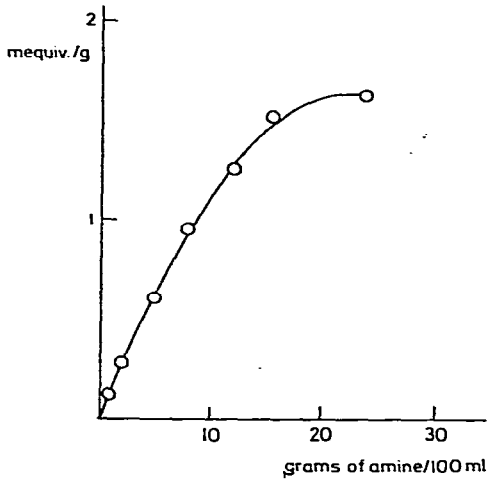
The effluent from column chromatography of proteins was evaluated by means of a through-flow "UV-analyzer 285 nm" (Development Works, Czechoslovak Academy of Sciences, Prague, Czechoslovakia); in the chromatography of nucleotides, a through-flow "Differential UV analyzer 254 nm" (Development Works) was used. The chromatographic analysis of amino acids was carried out with a Beckman Spinco 120 B amino acid analyzer with ninhydrin colorimetry. Ninhydrin colorimetry was also used in the chromatography of peptides, while non-peptidic chromo-

phores were recorded in advance with the through-flow "Differential UV analyzer 254 nm".

RESULTS

Ionogenic substitution

The substitution of hydrogen atoms with the DEAE group depends predominantly on the concentration of the amine used in the reaction mixture (Fig. 2). A slight increase in the degree of substitution may be achieved by extending the reaction temperature, by raising the temperature or by both. Capacities greater than 1.6 mequiv./g are difficult to reach; it is better to repeat the reaction with the product already substituted. However, as can be seen in Fig. 3, even if the reaction is repeated several times, it is virtually impossible to obtain capacities greater than 2.4 mequiv./g with the carrier under investigation.



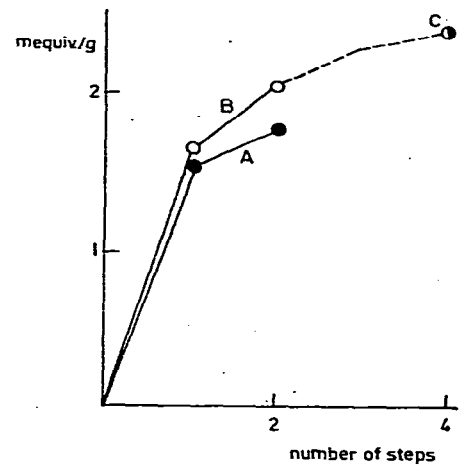


Fig. 2. Dependence of the capacity of DEAE-Spheron 300 on the ratio of N-2-chloroethyl-N,N-diethylammonium hydrochloride to the amount of water in the substitution reaction.

Fig. 3. Dependence of the resulting capacity of DEAE-Spheron 300 on repeated reaction with the previously substituted product. Sample A was prepared using a lower concentration of amine in the reaction mixture than samples B and C. In the case of C, only the capacity of the final product was determined.

Basic characterization of DEAE-Spherons possessing different ion-exchange capacities

Seven samples of DEAE-Spheron, the parameters of which are summarized in Table I, were prepared. Capacities for small ions (inorganic, *e.g.* cl^-) determined on a column (*cf.*, ref. 13) and from titration curves are practically the same. Capacities for small ions increase linearly with the nitrogen content (Fig. 4), in contrast to capacities for proteins. In the latter case the dependence rises steeply from very low values for unsubstituted Spheron even at a very small degree of substitution (Table I, sample 2); a further increase in substitution (samples 3–6) results in only a small rise in capacity. If proteins are sorbed batchwise, all the ionogenic groups probably do not play the same role, as is the case with small ions. The sorption of other macro-

TABLE I

PARAMETERS OF SAMPLES OF DEAE-SPHERON 300 WITH VARIOUS DEGREES OF IONOGENIC SUBSTITUTION

Samples 2–6 represent the main series of experiments. They were prepared in large amounts and systematically tested. Samples 1 and 7 were prepared in small amounts and tested sporadically.

Parameter	*Unit*	*Unsubstituted Spheron*	*Sample of DEAE-Spheron*						
			1	*2*	*3*	*4*	*5*	*6*	*7*
Carbon content*	%	55.68		55.23	56.30	54.52	58.06	58.55	
Hydrogen content*	%	7.56		7.76	7.89	8.30	8.61	8.95	
Nitrogen content*	%	0		0.40	0.80	1.71	2.29	2.78	
Moisture**	%	0		2.03	2.46	1.28	1.55	1.05	
Capacity for small ions by column method***	mequiv./g	0.03	0.11	0.26	0.60	1.23	1.63	2.05	2.20
Capacity for small ions by reading from titration curves	mequiv./g			0.25	0.58	1.23	1.63	2.04	
Capacity for serum albumin by batchwise method	mg/g	2–3		149	190	204	208	220	
Average working volume***,§	ml/g	3.95		3.88	3.60	3.55	3.23	3.13	
Unpenetrable volume, n***	ml/g	0.752					0.782		
Specific weight of unpenetrable mass in swollen state***	g/cm³	1.330					1.279		
Internal surface§§	m²/g	62					84		

* Averages from two analyses with an automatic analyzer.

** The moisture content of a predried sample after synthesis was determined by additional drying over phosphorus pentoxide at 0.1 mmHg.

*** Using a procedure described in ref. 13.

§ Valid for water. Increase by *ca.* 0.05 ml/g in 2 *M* hydrochloric acid, decrease by *ca.* 0.05 ml/g in 2 *M* sodium hydroxide.

§§ Determined by one-point method (*cf.*, ref. 13) after washing with acetone and thorough drying.

molecules on ion-exchange resins with an increased degree of substitution can proceed only with the participation of the best accessible functional groups on the external spacious parts of the macropores and on the bead surface. A large number of functional groups is sterically blocked by the proteins already sorbed. For this reason, further increases in the capacity for proteins is slow. One would be wrong to assume, however, that a low degree of ionogenic substitution is sufficient for chromatographic purposes. The capacity in the batchwise method is a result of an equilibrium process which is established only gradually towards the end, requiring several hours for completion. This batchwise process is more complicated than mere ionic sorption on the ion-exchange resin (as indicated by preliminary kinetic measurements), and is probably accompanied by association of the macromolecules. It cannot be completed in column chromatography, and capacities during the flow through the column are lower.

The titration curves of all derivatives of DEAE-Spheron over the whole range

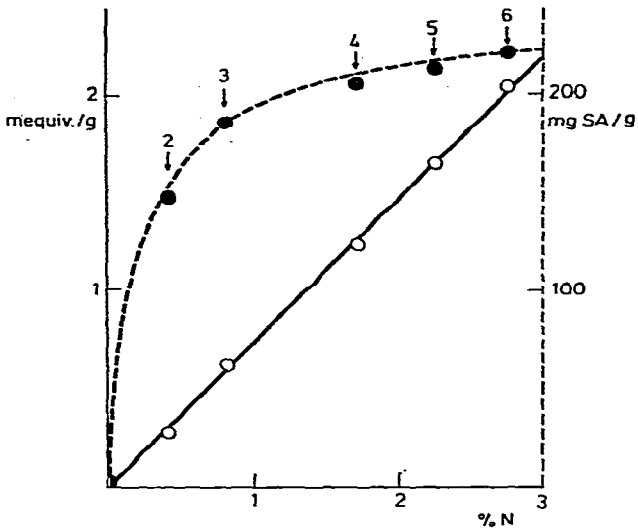


Fig. 4. Dependence of capacity on nitrogen incorporation in the ionogenic substitution of Spheron P-300 with the dimethylaminoethyl group. Numbers denote samples in Table I. ○—○, Capacity for small ions Cl^- in mequiv./g; ●- - -●, capacity for proteins in mg serum albumin/g.

of capacities are very similar (Fig. 5). Their shape does not seem to indicate the presence of a large number of groups having a pK different from DEAE. The flatter curves and lower apparent pK values compared with aliphatic tertiary amines are due to electrostatic effects, and probably also to the limited solvation of ionic groups on the gel surface.

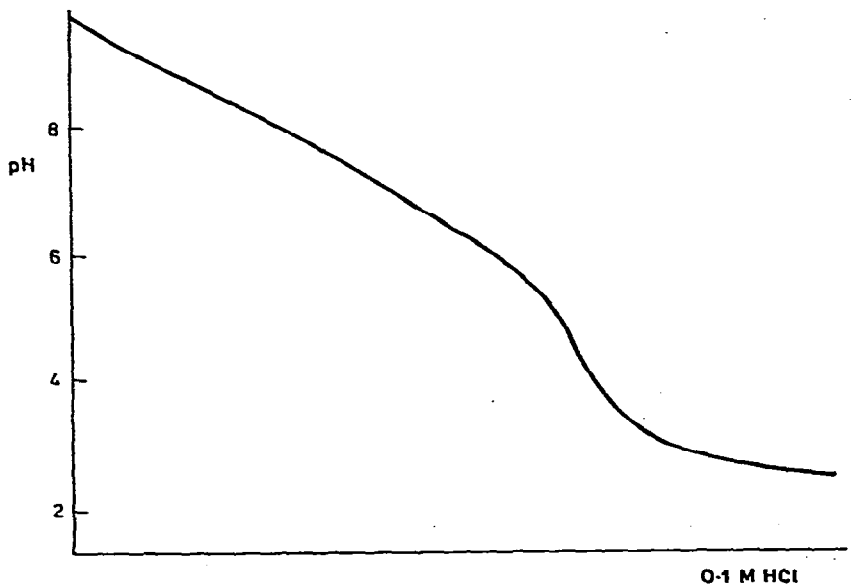


Fig. 5. Titration curve of DEAE-Spheron 300 having the capacity 1.63 mequiv./g. The ion-exchange resin (OH^-) was titrated in 1 M sodium chloride using an autotitrator.

Chromatographic characterization of DEAE-Spherons with different degrees of substitution

We investigated changes in the elution volume and peak shape in the chromatographic analysis of serum albumin on DEAE-Spherons of different capacities. The increase in capacities is accompanied by an increase in the retention volume *a* (measured in the chromatography of serum albumin by the gradient of the ionic strength, *cf.*, Fig. 6). At the same time, the width of the peak, *b*, also increases. On the other hand, *b*/*a* remains almost constant with further increase in substitution, after a steep drop with the initial degree of substitution.

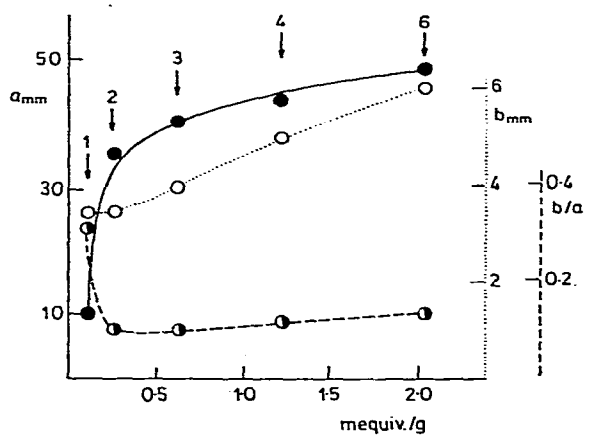


Fig. 6. Dependence of retention volume, *a* (●—●), peak width, *b* (○—○), at half-height and of their ratio, *b*/*a* (◑- - -◑), on the degree of ionogenic substitution of DEAE-Spheron 300 (mequiv./g) in the chromatography of human serum albumin. Numbers 1–6 denote the ion-exchange samples in Table I. Loading: 125 mg in 400 μl of buffer A (0.05 *M* acetic acid + ammonia, pH 8.0). Column (20 × 0.8 cm) equilibrated with buffer A; elution at 25° with linear gradient using 25 ml buffer A + 25 ml buffer B (buffer A, 1 *M* in sodium chloride), flow-rate, 65 ml/h. Pressure, 1–3 atm.

The dependence of the quality of chromatographic separation of pure proteins was checked on 30 mg of a synthetic mixture of lysozyme, chymotrypsinogen and serum albumin using the gradient of the ionic strength. The conditions were chosen so as to make the first two proteins separate with low resolution ,while the third protein would form an independent and completely separated peak. It can be seen in Fig. 7 that the ion-exchange resin with the maximum degree of substitution gave the best separation. One can also see a decrease in retention volumes for less substituted ion-exchange resins.

Another criterion used in the evaluation of these ion exchange resins was the chromatography of a natural complex mixture of proteins, namely, of blood plasma. The results obtained are summarized in Fig. 8, which shows that at a low degree of ionogenic substitution and with a 50 mg loading the ion-exchange resin is not able to adsorb some protein components, which are eluted as the first peak. At higher capacities, simultaneously with an increase in number of peaks, the height of the first peak representing the break-through of the proteins in the column decreases. The quality of the chromatogram (resolution) increases with increasing degree of substitution. An insignificant increase in the width of the last large peak (sample 6) does not

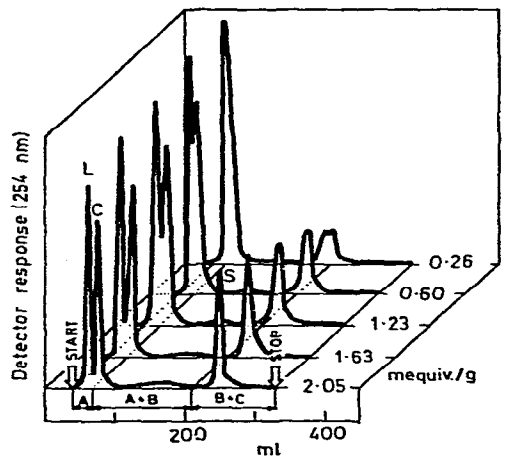


Fig. 7. Chromatography of a synthetic mixture of proteins on columns (20 × 0.8 cm) of DEAE-Spheron having various nominal capacities. A 5-mg amount of lysozyme L, 10 mg of chymotrypsinogen C and 15 mg of serum albumin S were dissolved in 0.4 ml of buffer A (0.005 *M* hydrochloric acid + Tris, pH 8.01), and applied on the column. The ion-exchange resins were equilibrated with the same buffer. The chromatographic analysis was performed using a gradient of the ionic strength: after isocratic elution with 25 ml of buffer A, the first linear gradient was used, 70 ml A + 70 ml B (0.2 *M* hydrochloric acid + Tris, pH 8.14), and eventually the second linear gradient, 60 ml B + 60 ml C (buffer B, 1 *M* in sodium chloride, pH 8.20). Flow-rate, 2 ml/min; temperature, 25°; pressure, 2–4 atm. Absorbancy at 254 nm was recorded continuously.

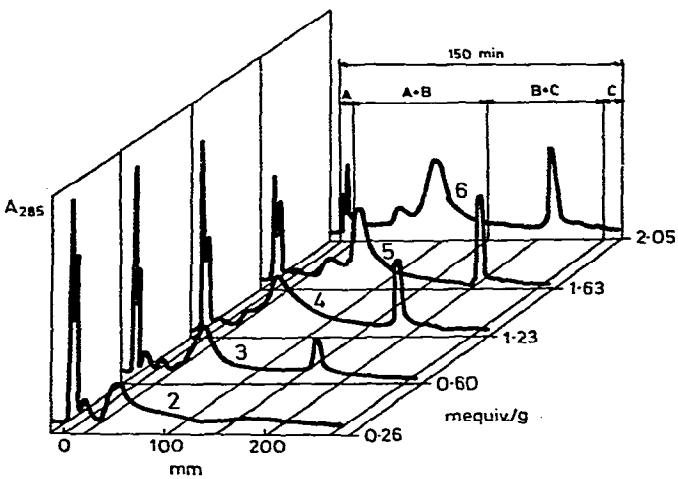


Fig. 8. Chromatography of human plasma with gradient of pH and ionic strength on DEAE-Spherons 2–6 having various degrees of ionic substitution (*cf.*, Table I). In all experiments, 50 mg of lyophilized plasma was dissolved in 0.4 ml of buffer A (0.025 *M* phosphoric acid + Tris, pH 8.48); after application to a column equilibrated with buffer A, the column was washed with 20 ml of the same buffer followed by a linear gradients 70 ml A + 70 ml B (0.5 *M* phosphoric acid + Tris, pH 3.49) and 60 ml B + 60 ml C (buffer B, 1 *M* in sodium chloride, pH 3.20). The column was eventually washed with 20 ml of buffer C. Other conditions as in Fig. 7, except that the experiments are listed in reverse order of nominal capacities for clarity.

indicate poorer separation: on the contrary, it suggests another possible separation, because the peaks do not represent homogeneous proteins, a large number of which have already been identified in the plasma.

In order to test ion-exchange resins for the separation of medium-molecular-weight compounds, we studied the chromatography of peptides, *i.e.*, of a commercial partial hydrolyzate of casein Trypton. The following conditions were used: column, DEAE-Spheron (20 × 0.8 cm) equilibrated with buffer A (0.05 *M* sodium citrate, pH 8); loading 2.5 mg in 0.2 ml of the same buffer; elution, at 25 °C with a linear gradient 50 ml A + 50 ml buffer B (buffer A, 1 *M* in sodium chloride) at a rate of 67 ml/h. detection, continuous recording of UV absorbancy at 254 nm (non-peptidic chromophores) followed by ninhydrin colorimetry in the amino acid analyzer. Trypton is a mixture of many peptides; it contains traces of dyes, and the complicated chromatograms obtained were therefore evaluated only subjectively with a result that the smallest number of peaks was obtained on the ion-exchange resin having the lowest degree of substitution, while the largest number was found for the anion-exchange resin of the highest degree.

The chromatography of amino acids in the amino acid analyzer was also examined. Fig. 9 shows the separation achieved using DEAE-Spheron of capacity 2.05 mequiv./g. The figure also shows quantities which define the parameters by means of which the ion-exchange resins were evaluated. The resolutions observed are summarized in Table II. The characterization of anion-exchange resins with respect to the separation of mononucleotides is illustrated by the chromatography of adenosine phosphates (Fig. 10). Again, the results show that the best separation is obtained on the anion-exchange resin having the highest degree of substitution.

DISCUSSION

The objective of this work was the preparation of an anion-exchange resin suitable for HPLC applications in biochemistry. For this purpose, the products obtained were tested in the chromatography of a great number of compounds, *viz.*, proteins (*cf.*, also ref. 15), peptides, amino acids, nucleotides, oligonucleotides (*cf.*, ref. 16) and sugars (*cf.*, ref. 17). The results obtained in this paper do not represent the optimal separations attainable using DEAE-Spheron. On the contrary, the conditions were so chosen that some peaks could be separated only with difficulty, because such cases were best suited for the evaluation of differences between the individual anion-exchange resins. In all the cases under investigation, the anion-exchange resin having the highest degree of substitution gave the best separation.

The effect of increasing capacity on gel structure is reflected in a decrease in the average working volume (Table I), which means that the degree of swelling decreases. This is an interesting paradox, since just the opposite behaviour is observed with ionogenically substituted xerogels, owing to an increase in repulsion between electrically charged groups. No explanation can as yet be offered for this effect.

In the preparation of DEAE-derivatives of natural matrices, such as cellulose, polydextran, agarose and starch, formation of basic groups of several types has been observed to various extents. In addition to the predominant diethylaminoethyl group (tertiary amine), the presence of strongly basic quaternary groups was also observed, reflected in titration curves (*cf.*, ref. 18). DEAE-Spheron, prepared as in *Methods*, is

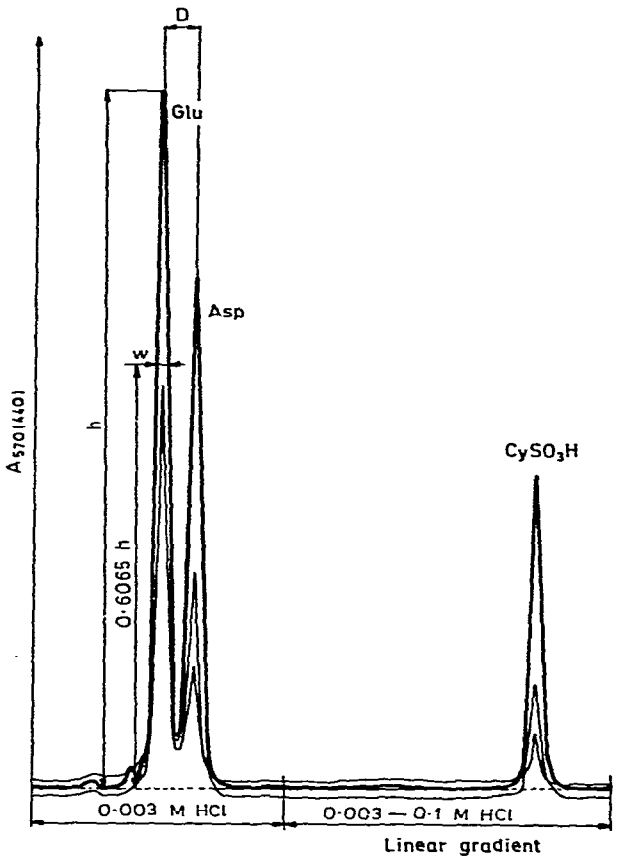


Fig. 9. Chromatography of amino acids on a column (20 × 0.8 cm) of DEAE-Spheron (Cl^-), capacity 2.05 mequiv./g. A mixture of 0.527 μmol of glutamic acid (Glu), 0.272 μmol of aspartic acid (Asp) and 0.127 μmol of cysteic acid ($CySO_3H$) dissolved in 0.4 ml of 0.003 *M* hydrochloric acid (pH 2.5) was introduced into the column. Elution was started with 20 ml of the same solution, followed by a linear gradient of 25 ml of the original solution + 25 ml of 0.1 *M* hydrochloric acid; the gradient was only 52% consumed. Flow-rate, 68 ml/h; temperature, 28°; pressure, 3–5 atm; detection, ninhydrin in an amino acid analyzer. h = Peak height; w = peak width in 0.6065 multiples of its height; D = difference in retention volumes at maxima of peaks under investigation.

TABLE II

RESOLUTION OF GLUTAMIC AND ASPARTIC ACIDS ON DEAE-SPHERON ELUTED WITH DILUTE HYDROCHLORIC ACID

Conditions as in Fig. 9. The chromatographic resolution, R_s, was calculated from $R_s = D(w_{Glu} + w_{Asp})^{-1}$. Cysteic acid was completely separated in all the experiments.

Capacity (mequiv./g)	*Chromatographic resolution (R_s)*
2.05	1.32
1.63	1.28
1.23	0.86
0.60	0.65*
0.26	—**

* The calculated value is only approximate.

** The value cannot be determined; glutamic and aspartic acids were almost unresolved forming one peak.

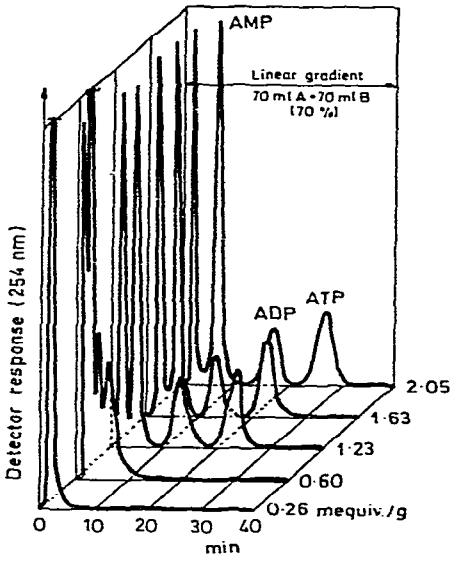


Fig. 10. Chromatography of a synthetic mixture of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) on a column (19.5 × 0.6 cm) of DEAE-Spheron of various capacities (0.26, 0.60, 1.23, 1.63 and 2.05 mequiv./g). Loading: 100 μl of solution containing 5 mg of each adenosine phosphate in 9 ml of water. Elution: a linear gradient of 70 ml of buffer A (0.1 *M* hydrochloric acid + Tris, pH 8.3) + 70 ml of buffer B (buffer A 0.2 *M* in sodium chloride, pH 8.3); only 70% consumed. Flow-rate, 147 ml/h. Evaluation: continuous absorbancy measurement at 254 nm; recording rates 120 mm/h.

a homoionic material and contains only one type of DEAE groups. The presence of quaternary groups cannot be seen in titration curves (Fig. 4); neither could it be observed in the chromatography of amino acids.

Of interest is the effect of ionogenic substitution on the hydrophobic properties of Spheron. It has been reported[12] that unsubstituted Spheron (containing no ionogenic groups) is a typically hydrophilic gel, the hydrophilicity of which is only slightly lower than that of the polysaccharide matrix. On the other hand, Spheron also possesses hydrophobic properties[13], so that it may be employed in the hydrophobic chromatography of some compounds[19–21]; in several cases it partly adsorbs proteins, especially at higher ionic strengths[21]. In this study we carried out experiments to elucidate the effect of the degree of ionogenic substitution of Spheron on this property. Since an anion-exchange resin was involved in this case, we chose lysozyme having an isoelectric point of 10.5–11, in order to suppress the possibility of electrostatic sorption. The experiment is demonstrated in Fig. 11. Lysozyme was first chromatographed on DEAE-Spheron columns equilibrated using a buffer with a low ionic strength, by elution with a linear gradient of the ionic strength. The chromatograms are not shown since the results obtained were normal for the chromatography of proteins on DEAE-Spherons. The peak width of lysozyme decreased with increasing degree of substitution, but only within narrow limits (7.5 to 3.8 mm); all the peaks were tall and narrow. In contrast, in the experiments shown in Fig. 11, in which the ion-exchange resins were equilibrated in advance using a buffer having a high ionic strength, ion-exchange resins with a low degree of substitution exhibited very considerable retardation and widening of the peak due to "salting-in"; the widening of the peak decreased until

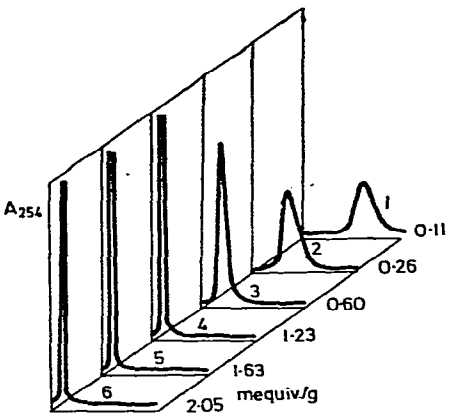


Fig. 11. Demonstration of the effect of the degree of ionogenic substitution on the intensity of salting-in of lysozyme on DEAE-Spheron 300. Column (20 × 0.8 cm) equilibrated with buffer B having a high ionic strength (0.05 *M* acetic acid + ammonia, pH 8.3, 1 *M* in potassium chloride). Loading: 2.5 mg of lysozyme in 0.2 ml of buffer B. Isocratic elution with the above buffer was carried out at 25° at a rate of 65 ml/h. Evaluation: continuous recording of UV absorbancy at 254 nm; chart speed, 120 mm/h. Numbers correspond to samples in Table I. In preceeding (not demonstrated) experiments same columns equilibrated with buffer A having a low ionic strength (0.05 *M* acetic acid + ammonia, pH 8.3) were used in the chromatography of 2.5 mg of lysozyme dissolved in 0.2 ml of buffer A by linear gradient elution with 25 ml A + 25 ml B under otherwise identical conditions (see text).

sample 4. The further narrowing of the peak in samples 5 and 6 is normal for an ion-exchange resin having a higher degree of substitution. It may be inferred from this experiment, therefore, that the weak hydrophobicity of Spheron 300 is almost balanced by hydrophillic ionogenic groups at degrees of substitution of 1.2 mequiv./g and higher. At a lower degree of substitution, the matrix of the otherwise hydrophilic gel may appear as being partly hydrophobic with respect to some proteins.

The DEAE-derivatives of Spheron prepared in this study were found to be very stable compounds; they have been repeatedly used in various chromatographic analyses without any deterioration of separations, although after each use they were recycled with 2 *M* sodium hydroxide and 2 *M* hydrochloric acid.

CONCLUSIONS*

Ionogenic substitution of the macroporous hydroxyalkyl methacrylate gel Spheron™ has no negative effects on its advantageous chromatographic parameters and its chemical and mechanical stability. The DEAE-derivatives with the highest degree of ionogenic substitution were found to be the most suitable for the chromatography of proteins, peptides, amino acids, oligonucleotides, nucleotides and sugars. A higher iogenic substitution suppresses the weak hydrophobicity of the predominantly hydrophilic Spheron matrix. DEAE-Spherons may be repeatedly used in chromatographic analysis, they do not lose their efficiency through regeneration and can be widely applied in the separation of many compounds of biochemical origin.

* *Note added in proof:* recently a paper by Svoboda and Kleinmann[22] appeared, illustrating a very good chromatographic preparative separation of radioactive nucleotides using the DEAE-derivative of Spheron 1000. The slightly modified DEAE-Spheron 300 was used by Vrátný *et al.*[23] for the excellent analytical separation of oligosaccharides.

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