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ION-EXCHANGE DERIVATIVES OF SPHERON

I. CHARACTERIZATION OF POLYMERIC SUPPORTS

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SUMMARY

In order to characterize matrices suitable for the preparation of ion exchangers, three commercially available types of glycol methacrylate macroreticular gels, Spheron™ P-100, P-300 and P-1000 (particle size, 20–40 μm), have been characterized by their bulk rate, degree of swelling, working volume and inner surface area. Prior to modification, the gels were extracted with dilute acid, dilute alkali, 8 *M* urea, pyridine and hot organic solvents. Extracted and dried Spherons have been characterized by elemental analysis, and by the determination of the inner surface area, exclusion limit, specific pore volume, the most frequent pore diameter, specific unpenetrable volume, number of unpolymerized double bonds and capacity for small ions. The particle-size distribution of Spheron P-300 has also been determined. Porosimetric data obtained by nitrogen sorption and desorption measurements are compared with those obtained by mercury porosimetry. The inner structure of Spheron is discussed in relation to the experimental results (electron microphotography and the course of thermal vacuum depolymerization, ion-exchange capacity after the highest attainable ionogenic substitution, unpenetrable volume and particle porosity). Extracted Spheron P-300 exhibits an advantageous, approximately level dependence of the height equivalent to a theoretical plate on the flow-rate within the range 25–280 ml/h. The suitability of a Spheron matrix for the preparation of ion exchangers is demonstrated.

INTRODUCTION

Hydrophilic slightly cross-linked gels, based on the copolymerization of glycol methacrylates with alkylene dimethacrylates, were used by Wichterle and Lím[1] for applications in medicine. The hydrophilic character of this type of macromolecular matrix, combined with a high chemical stability and mechanical strength, led Čoupek

and co-workers[2,3] to the development of a macroreticular spherical form of hydroxyalkyl methacrylate gel (called Spheron™), which was designed for gel permeation chromatography (GPC)[4]. The gel was found to be suitable not only for GPC[5–8], but also as a carrier in the immobilization of enzymes[9–14] and in affinity[15–21], gas[22–24], chelation[25,26] and adsorption (hydrophobic)[27–29] chromatography; it is now commercially available*.

The advantageous chromatographic properties of macroporous hydroxyalkyl methacrylates and the need to develop new types of ion exchangers resistant to the sorption of technical enzymes from cultivation liquids have led to the use of Spheron as a matrix in ionogenic substitution. Procedures have been suggested for the preparation of Spheron ion exchangers possessing both classical[30–33] and dipolar[34,35] ionogenic groups. In a previous paper[36] we showed that ion-exchange derivatives of the former group[30–33] are suitable for the chromatography of biopolymers and promising for the application of high-performance liquid chromatography (HPLC) to the separation of these compounds. We also demonstrated the advantages of these derivatives in the chromatography of technical enzymes[37] and sugars[38].

This paper concentrates on problems related to the characterization and choice of a Spheron matrix suitable for the preparation of ion-exchange materials. As with a forthcoming paper on DEAE-Spheron[39], the properties of this new type of ion exchangers are discussed in some detail.

EXPERIMENTAL

Materials

Spheron P-100, P-300 and P-1000 (nominal particle size range, 20–40 μm) were obtained from Lachema, as were the reagent grade chemicals used. Organic solvents were redistilled before use. Distilled water was deaerated. Serum albumin (98%) was obtained from Imuna (Šarišské Michalany, Czechoslovakia). Chymotrypsinogen was prepared by five recrystallizations of a product supplied by Léčiva (Prague, Czechoslovakia). Egg-white lysozyme was obtained from Worthington (Freehold, N.J., U.S.A.).

Methods

The dry residues contained in the Spherons were determined by drying to constant weight *in vacuo* (0.1 mmHg) over NaOH and H_2SO_4. The bulk rate of dry Spheron and its inverse value, the bulk volume, were ascertained by weighing a stabilized Spheron volume in a graduated cylinder after the material had been made to settle by soft tapping on the cylinder. Decantation was carried out in wide graduated cylinders by triple sedimentation of a suspension of the gel (stirred with ten times its volume of water) which had been deaerated *in vacuo* before sedimentation**. The working volume of the swollen gel (dry weight, 1 g) was determined in the chromato-

* In Europe, Spheron™ is produced by Lachema, (Brno, Czechoslovakia); in West European countries it is distributed by Koch-Light (Colnbrook, Great Britain). In the U.S.A., Spheron™ is produced by Hydron Labs. (New Brunswick, N. J.) and distributed by Realco (New Brunswick, N.J.).

** Gels of the Spheron type retain air in the pores if wetted with a liquid. It is therefore necessary to remove air from the suspension by means of a brief evacuation prior to decantation or chromatographic application.

graphic column (diameter, 0.8 cm; length, 25 cm) at a flow-rate of water of 75 ml/h. Swelling was characterized as an increase in volume above the bulk volume according to:

$$\text{Swelling (\%)} = \frac{\text{working volume} - \text{bulk volume}}{\text{bulk volume}} \cdot 100 \quad (1)$$

Prior to modification, Spheron was extracted in turn with 0.1 *M* sodium hydroxide, 0.1 *M* hydrochloric acid, 8 *M* urea and pyridine, and then by successive boiling with methanol. benzene and acetone. The gels were freed from the residues of the solvent by drying at a water pump, followed by drying at an oil pump to constant weight.

The exclusion limits of Spheron were determined by chromatography of standard polydextrans (Pharmacia, Uppsala, Sweden) on an L-Chrom 50 liquid chromatograph (Laboratory Instruments Works, Prague, Czechoslovakia), using 50 × 0.8 cm columns and water as the eluent (flow-rate, 1 ml/min). Detection was by means of a Waters 403 differential refractometer. The inner surface area of Spheron[23] was measured by using the dynamic method of air sorption at liquid-nitrogen temperature in a simplified apparatus[40], and by comparison with standards having a known inner surface area (aluminium oxide, titanium dioxide). The hysteresis curves for nitrogen sorption and desorption in the pores of gels P-300 and P-1000 were determined with a Sorbtomatic Type 1800 apparatus (Carlo Erba, Milan, Italy). Mercury porosimetric measurements of Spheron within the range 0–100 MPa were carried out using a Carlo Erba Model 56 apparatus; the results obtained by the recording manometer were evaluated by means of a Tesla 200 computer.

The unpenetrable volume, n (in ml/g), of the Spheron matrix was assessed pycnometrically at 25° by use of eqn. 2:

$$n = \frac{V}{Q_1 \cdot Q_2}(Q_1 + Q_2 - Q_3) \quad (2)$$

Q_1 = weight of the liquid in the pycnometer (volume $V = Q_1/s'$, where s' is the specific weight of the liquid), Q_2 = weight of *ca.* 0.25 or 0.5 g of Spheron introduced into the dry pycnometer and Q_3 = weight of the same Spheron and of the liquid after swelling in a small amount of water, after deaerating twice in an evacuated desiccator and after bringing the liquid in the pycnometer up to the mark. The reciprocal value of n is the specific weight, s (in g/ml), of the unpenetrable matrix mass in the swollen state:

$$s = \frac{1}{n} \quad (3)$$

The capacity for small ions was determined on a chromatographic column (25 × 0.8 cm) containing 1 g of Spheron. The gel in the column was sedimented in a flow of water (75 ml/h). The volume of the column determines the working volume of the gel. In the determination of the ion-exchange capacity, *ca.* 5 ml of *ca.* 2 *M* hydrochloric acid were placed above the Spheron volume: 4 ml were forced through the column and the rest after 5 min. The procedure was repeated twice, and the column in the H^+ form was then washed with water until the effluent was neutral.

For the titrimetric determination of the capacity of Spheron, 5.00 ml of a 0.1 *N* sodium hydroxide solution were poured on top of the column. Simultaneously another 5.00 ml of the sodium hydroxide solution were placed in a flask and diluted with 20 ml of boiled-out water for use in a blank test. The titrating solution was forced through the column and washed with another 20 ml of boiled-out water into a titration flask protected against contamination by carbon dioxide. The effluent was then transferred to another titration flask; water was pumped through the column, and another 25 ml of the effluent were collected in order to determine the degree of hydrolysis of the cation exchanger in the Na^+ form. The solutions in the three flasks were then titrated in the same way. The capacity was calculated from the difference between the blank test and the determination, with the hydrolysis value being added.

The sorption of proteins in the chromatographic column was assessed by applying 5 mg of serum albumin, 1.25 mg of chymotrypsinogen or 1.25 mg of lysozyme dissolved in 0.2 ml of solvent to a column (25 × 0.8 cm) containing 3.35 g (dry weight) of Spheron equilibrated with the respective solvent (water or 0.02 *M* sodium acetate–acetic acid, pH 5.5). The protein content in the effluent was determined spectrophotometrically at 280 nm. The traces of carboxyl groups in Spheron were esterified with diazomethane in a methanolic solution (200 ml per 10 g of Spheron), which was prepared from 150 ml of methanol and 50 ml of a diethyl ether solution of diazomethane obtained from the reaction between 5 g of N-nitrosomethylurea in 20 ml of 40% potassium hydroxide solution.

The flow-rate characteristics of Spheron columns (20, 50 and 120 × 0.8 cm) were determined on a L-Chrom 50 liquid chromatograph from the dependence of the theoretical plate height on the flow-rate within the range 20–300 ml/h.

The number of pendant double bonds possessed by Spheron was ascertained by laser Raman spectrophotometry using a Coderg LRDH 800 apparatus, according to Štokr *et al.*[41]. Elemental analysis was carried out by using a Perkin-Elmer automatic analyzer after complete disintegration of the gel particles in a vibration mill; this condition is necessary for reproducible results. Electron microscope photographs were obtained with a JSM 35 JEOL apparatus.

RESULTS

Commercially available Spherons, chosen according to the published data[4], were characterized using the data summarized in Table I. While both the bulk and

TABLE I

BASIC DATA CHARACTERIZING SPHERON GELS BEFORE TREATMENT

Quantity	*Spheron*		
	P-100	*P-300*	*P-1000*
Bulk rate (g/ml)	0.608	0.545	0.337
Bulk volume (ml/g)	1.645	1.835	2.970
Dry residue (%)	96.6	95.9	92.2
Swelling (%)	115	114.5	56.5
Working volume (ml/g)	3.55	3.95	4.65
Inner surface area (m^2/g)	13.5	42	139

working volumes increase in the order P-100 < P-300 < P-1000 together with the inner surface, the degree of swelling decreases.

The Spherons contained extractable residues of the reaction components used in their production, which may have an unfavourable effect on the sorption of biopolymers. As a precaution, the gels were extracted with solvents as described under Experimental. The resulting material was tested for its sorption of proteins in water and in buffers at low ionic strengths. After removal of the trace amounts of free carboxyl groups by treatment with diazomethane, we found a 100% recovery of pepsin, serum albumin, chymotrypsinogen and lysozyme, which represent both acidic and basic proteins. With some proteins, an increase in the ionic strength may lead to their retention.

Decanted, extracted and dried Spheron gels were characterized by their particle-size distribution, which for Spheron P-300 is shown in Fig. 1, and by the parameters summarized in Table II. The exclusion limits of dextrans found for the

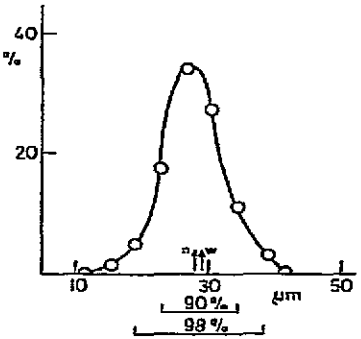

Fig. 1. Grain-size distribution of Spheron P-300 prepared for ionogenic substitution. Maximum scattering of 90% of the material occurs of 23.1–34.6 μm, of 98% of the material at 19.2–38.5 μm. Number average, n, of the grain size, 28 μm; weight average, w, 29 μm.

commercially available gels extracted by us, and compared with data[4,6,9] for gels prepared on a laboratory scale and data of an American producer[42], are summarized in Table III.

The inner structure of Spheron particles was investigated by mercury porosimetry (Fig. 2) and by nitrogen sorption (Fig. 3). The dependence of $dV/d\log r$ (where V is the specific pore volume and r is the pore radius) against $\log r$ obtained by the two methods for Spherons P-100, P-300 and P-1000 exhibits a marked maximum between 100 and 300 Å. Fig. 2 shows the very narrow maxima observed with Spheron P-300 and P-1000 corresponding to pores having a diameter of *ca.* 1800 Å. In the nitrogen-desorption measurements (Fig. 3) these maxima are no longer seen; the method, based on measurements of the magnitude of the inner surface of pores, is not very sensitive for large pores. The occurrence of such maxima may be related to a partial destruction of the inner structure of the gel particles owing to the pressure of mercury; however, an interpretation based on the presence of macropores into which mercury can more easily penetrate seems more likely. At pressure above 900 atm, the differential curve indicates mechanical destruction of P-300 and P-1000.

TABLE II

PARAMETERS OF SPHERON GELS AFTER EXTRACTIONS AND DRYING

None of the Spherons analyzed contained nitrogen or ashes.

Quantity	*Spheron*		
	P-100	*P-300*	*P-1000*
Carbon content (%)	56.46	55.68	56.56
Hydrogen content (%)	7.19	7.56	7.72
Inner surface area * (m^2/g)	67	55	155
Inner surface area(BET) ** (m^2/g)		48	94
Exclusion limit *** (dalton · 10^3)	100	501	1000
Specific pore volume § (ml/g)	0.534	0.601	1.69
Specific unpenetrable volume §§ (ml/g)	0.724	0.752	0.761
Specific weight of unpenetrable mass in swollen state §§§ (g/ml)	1.385	1.330	1.310
Amount of unpolymerized double bonds † (mol. %)	7.5	19.3	0
Cation-exchange capacity for small ions (mequiv./g)	0.04	0.04	0.04

* Determined using the Kljačko–Gurvič method[40].

** Determined from the desorption isotherms of nitrogen.

*** Determined by using standard polydextrans.

§ Measured by mercury porosimetry within the range 75–75 · 10^3 Å.

§§ For distilled water, n_{H_2O}.

§§§ For distilled water, s_{H_2O}.

† Determined according to ref. 41 and expressed in mol. % related to the original amount of the cross-linking agent.

TABLE III

COMPARISON OF THE MOLECULAR-WEIGHT EXCLUSION LIMITS OF EXTRACTED GELS WITH DATA PUBLISHED FOR UNTREATED GELS

Spheron	*Data from refs. 4, 6 and 9 and the American producer[42] (daltons · 10^3)*	*Data of Lachema[43] and of the European distributor[44] (daltons · 10^3)*	*Data for extracted Spherons in this paper (daltons · 10^3)*
P-100	100	70–250	100
P-300	300	260–700	501
P-1000	1000	800–5000	1000

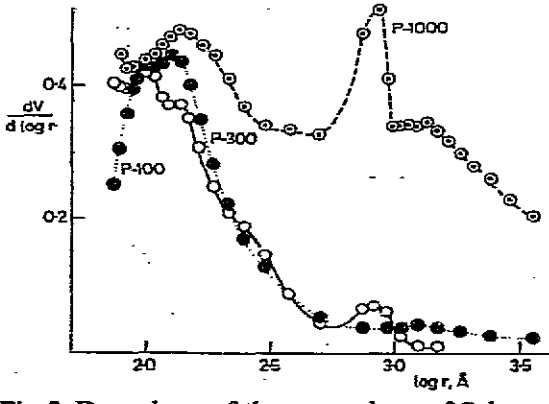

Fig. 2. Dependence of the pore volume of Spheron on pore radius obtained from mercury porosimetric data. The plot shows only part of the measurements performed.

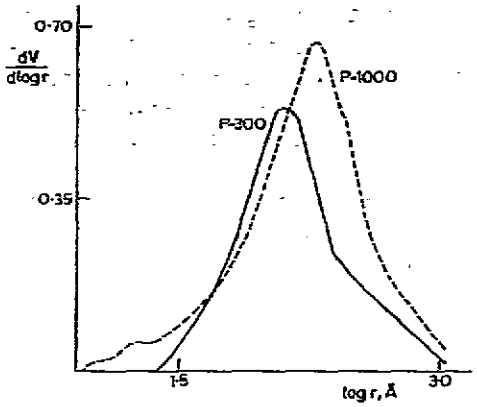

Fig. 3. Dependence of the pore volume of the Spheron gels on pore radius obtained from measurements of the desorption isotherms in the low-temperature adsorption of nitrogen in the Sorbomatic apparatus.

A comparison of the results obtained with the three Spheron gels in the dry state is shown below:

Diameter (Å) of the most frequent pores determinated by	*Speron P-100*	*P-300*	*P-1000*
(a) mercury porosimetry	200–250	180–280	
(b) desorption of nitrogen		250	370

The similar values of the maximum pore frequency for gels P-100 and P-300 (diameter *ca.* 200 Å) do not indicate, *cf.* the different swelling of the gels (Table I), that the exclusion limits in the gel–solvent contact are similar (Table II).

The unpenetrable gel volume (Table II) is a measure of the size of that part of the Spheron matrix which is formed by the organic mass or is so densely cross-linked that it is inaccessible even to the solvent in which the gel has been swollen. The use of solvents having different molecule sizes provides information on this parameter. The data for water as the main solvent in the ion-exchange chromatography of biopolymers are given in Table II. Similar measurements on Merckogel OR-2000 using various solvents were performed by Saitoh *et al.*[45].

The cross-linking polymerization process results in a polymer containing a fraction of the double bonds of the cross-linking agent. This fraction was determined quantitatively by laser Raman spectrometry: for Spheron P-1000, P-100 and P-300 it is undetectable, 7.5 mol.% and 19.3 mol.%, respectively. Since the composition of the three copolymers is similar, the content of the double bonds is probably related to the kinetics of the polymerization reaction. The detectable double bonds are probably enclosed in the polymeric matrix of the gel, and thus also inaccessible to low-molecular-weight compounds. Basing on the finding that no negative effect of the double bonds on the sorption of chromatographed compounds could be observed, one may infer that under normal conditions these bonds are not reactive.

The ion-exchange capacity of extracted Spheron for small ions (Table II) is small (0.04 mequiv./g), lying almost at the limit of detection of the method used.

These ionogenic groups may be ascribed mainly to the carboxyl groups arising from methacrylic acid and present in trace amounts in the monomers used. The undesired carboxyl groups can be readily removed by use of specific alkylating agents (*e.g.*, diazomethane or trialkyloxonium compounds). However, chromatographic analysis on ion-exchange derivatives based on these matrices is not affected by the presence of trace amounts of carboxyl groups.

The dependence of the separation efficiency in HPLC on the flow-rate of the eluent for columns 8 mm in diameter and of various length is shown in Fig. 4. Slurry packing was the best of all the known methods, but is fully satisfactory only with columns 20–50 cm long, the flow characteristics of which are identical. A longer column packed in the same way exhibited a lower efficiency.

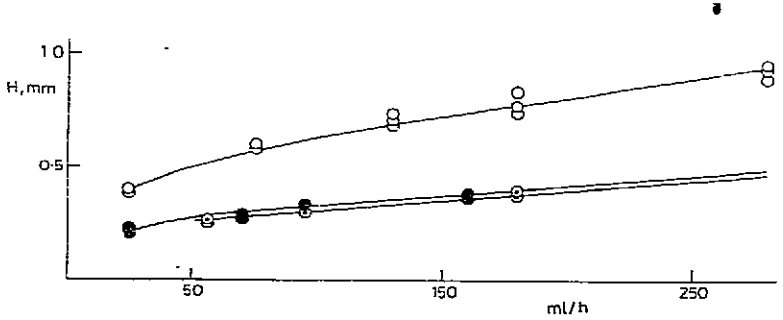

Fig. 4. Dependence of the height equivalent to a theoretical plate, H, of columns of various length on the flow-rate (ml/h) of water using Spheron P-300 as packing (32–40 μm). Glucose was the test standard at 25 °C. Columns: ○, 100 × 0.8 cm; ●, 50 × 0.8 cm; ○, 20 × 0.8 cm.

DISCUSSION

The criteria used when choosing the ion-exchange column supports for HPLC of biopolymers discussed in ref. 36 must of course hold for their matrix, in the first place. Hydroxyalkyl methacrylate gels of the Spheron type contain a large amount of nonionogenic hydroxyl groups, the reactivity and solvation of which are analogous to primary aliphatic alcohols. These gels are sufficiently hydrophilic for the chromatography of biopolymers, but are more hydrophobic than cellulose, agarose or polydextran. From refs. 28, 29 and 46, it can be inferred that hydroxyalkyl methacrylate sorbents exhibit good properties in solvophobic chromatography. Their macroporosity, readily controlled within wide limits, and large inner surface with reactive hydroxyl groups make them a good matrix for the preparation of ion exchangers. The hydrophilicity of the gel is obviously further increased by ionogenic substitution. The possibility of preparing the gels in the bead form and their mechanical strength and flow parameters at high rates and pressures characterize these gels as very suitable supports for modern HPLC.

The high chemical stability of hydroxyalkyl methacrylate gels has already been pointed out in earlier papers[3,36]. These materials are amongst the most chemically stable of the known organic carriers used as supports in biochemical applications. No perceptible changes could be observed after the basic material and ion-exchange derivatives had been subjected to long-term treatment with alkalis and acids.

The densely cross-linked insoluble microstructure, which prevents the penetration of large molecules, forms microspheres which are separated from solution in the suspension polymerization and joint each other to form the final macrospheres (beads)[36]. A real view of the cross-section of a Spheron bead is shown in the electron scanning microphotograph in Fig. 5. Further evidence in support of the described inner structure of the macroporous Spheron particles is provided by the results of preliminary experiments on thermal vacuum depolymerization which are summarized in Fig. 6. The sudden collapse of the Spheron beads to a microscopic dust indicates

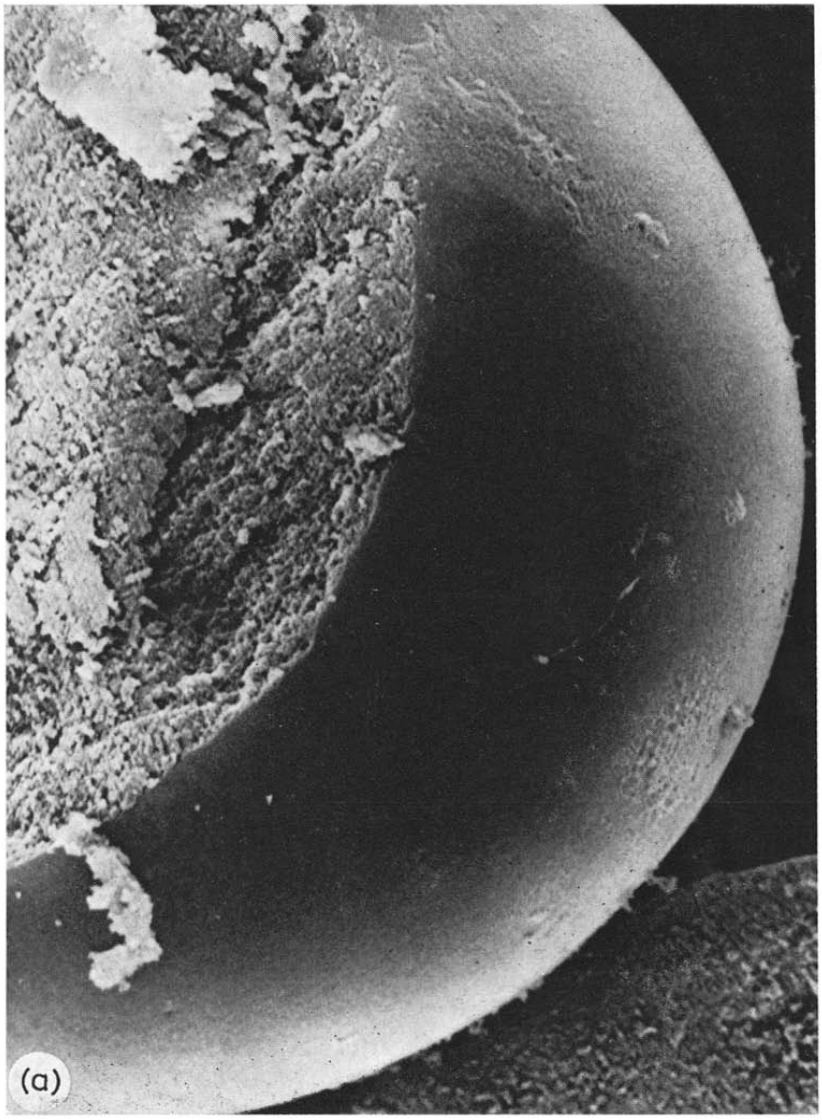

Fig 5a. Electron scanning microphotograph of a cut through a dry Spheron P-300 macrosphere.

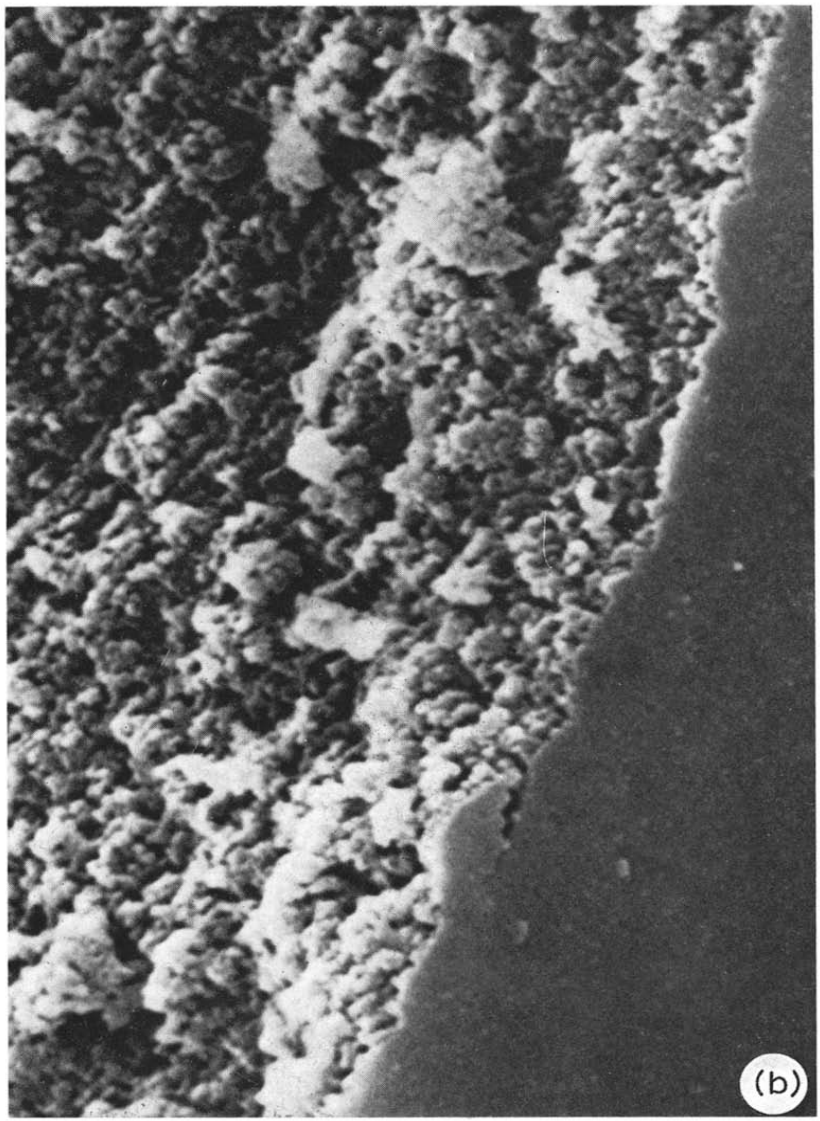

Fig. 5b. Detailed insight into the agglomeration of microspheres in dry Spheron P-300 particle as shown by electron scanning microphotography.

that their macrostructure consists of agglomerated microspheres, weakly interconnected through polymeric bridges[47].

The basic view of the macro- and micro-structure of Spheron described above should be made more exact from the standpoint of the measured capacities of Spheron ion exchangers for small ions[36,39]. Thus, for example from extracted Spheron P-300 characterized in Table II, it would be possible to prepare ion exchangers having a capacity of up to 2.2–2.4 mequiv./g by replacing the hydrogen atoms by diethylaminoethyl groups[39]. Assuming that the functional groups are arranged parallel to

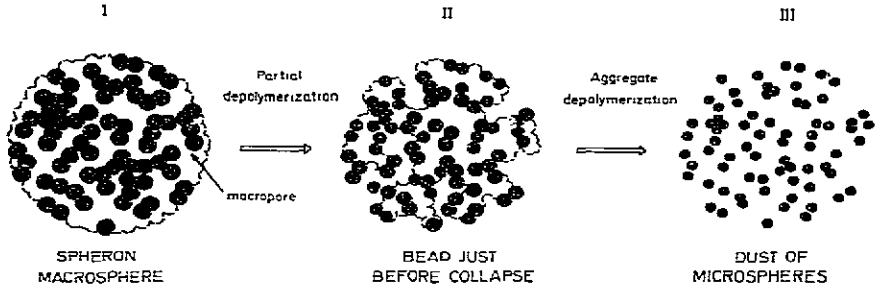

Fig. 6. Schematic view of the macrostructure of Spheron beads (I) based on thermal depolymerization in high vacuum. Gradual treatment at high vacuum (10^{-5} Torr) and elevated temperature (270–280°) leads to surface depolymerization of cross-linked microspheres and to the reduction in size. This also results in a weakening of the connections of the macrostructure (II) and in its sudden collapse, which yields microscopic dust (III).

each other so as to project from the inner surface directly into the bulk of the macropores, and that the diethylamino group in the *trans–trans* conformation has an effective diameter of 8.5 Å, the occupied area for a capacity of 2.3 mequiv./g would amount to $(8.5\ Å)^2 \cdot 10^{-20}\ (m^2/Å^2) \cdot 6 \cdot 10^{23} \cdot 2.3 \cdot 10^{-3}$ equiv./g $\approx$ 1000 m^2/g. However, the measured inner surface areas (Table II) are lower by an order of magnitude. On the other hand, it should be borne in mind that the surface-area measurements were carried out with the gel in the dry state and that the cross-linked microspheres in contact with the solvent are swollen and the whole particle increases in volume. This probably permits a larger participation of the polymeric substance in the ionogenic substitution with small molecules.

The calibration graph for Spheron P-300 (Fig. 7) allows one to read off the particle-porosity value[48] for glucose (1.3 ml/g). The particle-porosity value calculated from the unpenetrable volume for water is 1.48 ml/g. This difference is obviously connected with the molecular dimensions of the two penetrating compounds. Using the composition of the polymerization batches, it can be calculated that Spheron P-300 contains on the whole *ca.* 6.0 mequiv./g of hydroxyl groups. From the ratio of the maximum capacity of 2.4 mequiv./g for DEAE-Spheron[39] to the total number of hydroxyl groups it follows that substitution may consume *ca.* 40% of the hydroxyl groups present in the polymer. Using the known amount of conversion of the hydroxyl groups, it is possible to calculate the particle porosity (0.9 ml/g) for chloroethyldiethylamine hydrochloride used in the ionogenic substitution. Consequently, in the bulk penetrable to water (1.48 ml/g), 60% of the hydroxyl groups have been consumed. Together with the larger molecular size of the substitution agent compared with water, the reason for the existence of the unreacted moiety may also be the lower reactivity of hydroxyl groups sterically hindered by the polymeric matrix.

The rigidity and very good hydrodynamic properties of Spheron particles in HPLC are demonstrated by the dependence of the theoretical plate height on the flow-rate within the range 25–280 ml/h (Fig. 4). Of course, together with the physical properties of the packing and the uniformity of the particle size, an important factor is also the way in which the column is packed, and, last but not least, the way in which the column has been constructed. A characteristic feature of Spherons is the

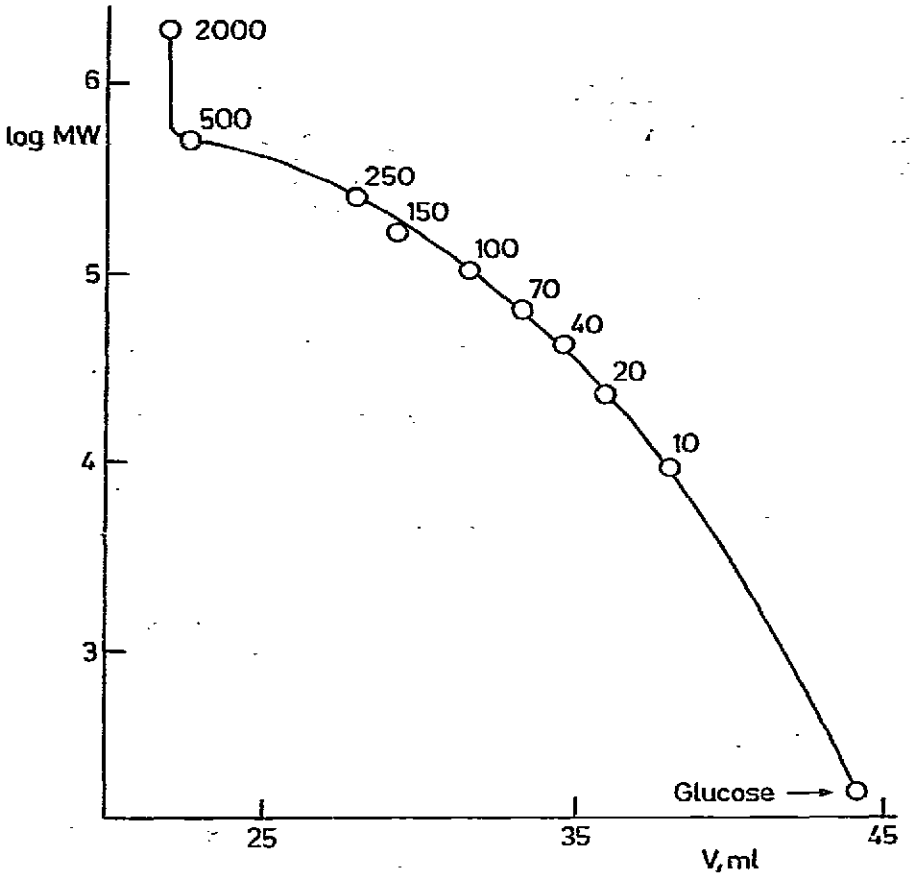

Fig. 7. Calibration curve of Spheron P-300 (32–40 μm) on a column (120 × 0.8 cm) using glucose and polydextran fractions. The dry gel content was 17 g. Figures at the individual points on the curve denote molecular weights of the fractions in thousands of daltons.

approximately level course of the *H vs. V* dependence (in the GPC mode), which allows one to work at extremely high flow-rates while preserving a satisfactory efficiency.

Preliminary tests[36] and the work reported here have shown that Spheron can be used in the preparation of all known types of ion exchangers from the available ion-exchange derivatives of cellulose, polydextran and agarose. Spheron is superior to these matrices made from natural materials in respect of its mechanical properties, its column-flow parameters, its volume stability with changing ionic strength and pH, its resistivity towards organic solvents, enzymes and microorganisms and its overall chemical stability.

The Spheron macrostructure can be characterized as an aerogel; in chromatography its macropores are filled with pure solvent, allowing one to obtain much higher diffusion rates of compounds being separated into the bulk of the macropores, and thus also to the ionogenic groups, than a swollen xerogel of other types of matrices. This is what makes Spheron and its derivatives suitable not only for the chromato-

graphy of macromolecular biopolymers, but also of their fragments and other low-molecular-weight compounds.

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