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ION-EXCHANGE DERIVATIVES OF SPHERON

V*. SULPHATE AND SULPHO DERIVATIVES

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SUMMARY

Using various reactions, strongly acidic derivatives of Spheron of the sulphate and sulpho types were prepared with nominal capacities between 0.37 and 2.0 mequiv./g. They were characterized by their capacities for small ions, by their static and dynamic capacities for proteins, by elemental analysis, by their working volumes and specific internal surface areas. The differences between the nominal capacities calculated theoretically from the sulphur contents and the measured values are discussed, as are the relationships between the nominal capacities for small ions and the static and dynamic capacities for proteins. The chromatographic properties of the cation exchangers are illustrated by the separation of an artificial mixture of proteins (serum albumin, chymotrypsinogen and lysozyme) at various pH values, and of a natural mixture of egg-white proteins. The separation of active enzymes is also reported. Sulphobutyl-Spheron was tested also as a catalyst for the esterification of alcohols.

INTRODUCTION

In this series of papers dealing with Spheron ion-exchange derivatives bearing classical functional groups we have previously reported the characterization of the polymer matrix[1], the preparation and properties of DEAE derivatives[2], carboxylic ion exchangers[3] and phosphate ion exchangers[4]. The whole series was preceded by an introductory paper[5] explaining the reasons for such investigations. In addition a series of papers on the application of Spheron ion exchangers has also been published (cited in ref. 4).

* For Part IV, see ref. 4.

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The aim of the penultimate paper in this series is to describe the preparation and properties of sulphate and sulpho derivatives of Spheron and their utilization in the chromatography of enzymes and other proteins, and in catalysis.

EXPERIMENTAL

Materials

Spheron 300 and 1000 and proteins used in the chromatographic tests were the same as the starting materials in ref. 4. Spheron 100 was obtained from Lachema (Brno, Czechoslovakia). The properties of Spheron 100, 300 and 1000 are described in ref. 1. The sources of all the other chemicals and of the cellulolytic enzyme sample were as in ref. 4. Chemically identical macroporous spherical copolymers of 2-hydroxyethyl methacrylate are now manufactured under the trade name Separon HEMA[6] by Laboratory Instrument Works, Prague with special emphasis on high-performance applications.

Preparation of ion-exchange derivatives for chromatography and catalytic experiments

Preparation of cation exchanger by use of chlorosulphonic acid according to eqn. 2 (Results and discussion). A 20-g amount of dried Spheron 300 (30–40 μm) was suspended in 210 ml of dried ethyl acetate and cooled to −20°C. Chlorosulphonic acid (50 g) was dissolved in 150 ml ethyl acetate, cooled to −20°C and added to the Spheron suspension, with cooling so that the temperature did not exceed −16°C. After stirring for half an hour, the Spheron was washed with 200 ml ethyl acetate, then with acetone, ethanol, water and acetone and dried. It contained 4.73% S and did not contain chlorine.

The sulpho derivative may also be prepared without cooling by treating the glycidyl derivative of Spheron with sodium bisulphate according to eqn. 4 (Results and Discussion). To 0.3 g glycidyl-Spheron (for preparation see ref. 4), 1.5 g $NaHSO_3$ and 3 ml water were added, and the mixture was thermostatted in a closed vessel at 98°C for 4 days. The gel was then thoroughly washed with water, left to stand for 20 min with 2 *M* NaCl, washed with water, ethanol, acetone and diethyl ether and dried. The product contained 3.04% S.

A suitable method for the preparation of Spheron ion exchangers consists in the reaction with cyclic sulphones according to eqn. 5 and as illustrated by data in Table I and Fig. 1.

Chromatographic methods and the evaluation of fractions were described in ref. 4 and in preceding papers[1–3]. Testing of the activity of cellulolytic enzymes which decompose filter paper was described in ref. 4.

Testing of the catalytic activity of Spheron SB under static conditions

n-Butanol was esterified with acetic acid in a three-neck 250-ml flask provided with a stirrer, a thermometer and an adapter for azeotropic removal of water by distillation. The reaction mixture contained 1 mol acetic acid, 1.2 mol *n*-butanol and 3 g Spheron SB 300, particle size 63–100 μm and exchange capacity 1.97 mequiv./g. Esterification carried out with the boiling reaction mixture was complete within 80 min, as soon as the vapour temperature increased from 93°C (b.p. of azeotropic *n*-butanol–water) to above 120°C.

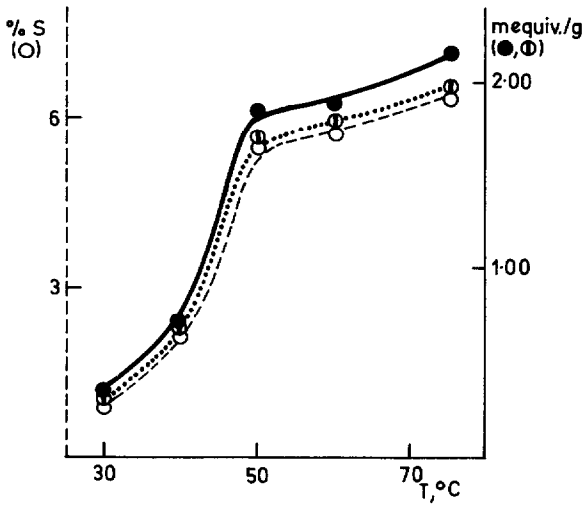


Fig. 1. Effect of the reaction temperature on the nominal capacity of the cation exchanger in the reaction of Spheron 300 (40–63 μm) with 1,4-butanesultone. The reaction procedure was that in Table I, except that each time 50 ml (66.9 g, 482 mmol) butanesultone and 80 ml of 12.5 *M* NaOH, *i.e.*, 1000 mmol, were used. The reaction time was 1 h in each case. Open circles denote the sulphur content according to elemental analysis, full circles the nominal capacity determined by transferring the ion exchanger (H^+) into excess of alkali and back-titrating with acid using phenolphthalein; ⊕, the theoretical nominal capacity calculated from the sulphur content.

RESULTS AND DISCUSSION

Preparation of cation exchangers and their analysis

Strongly acidic derivatives of Spheron[1,5] (Separon HEMA)[6] may be divided into two groups: (a) sulphate derivatives in which sulphur is bound to the Spheron matrix through oxygen; (b) sulpho derivatives in which sulpher is bound through carbon; it is expected that type (b) will be more stable chemically. Sulphate derivatives (a) to be used for chromatographic purposes[5] may be most simply prepared by the reaction of sulphur trioxide dissolved in concentrated sulphuric acid with hydroxy groups of Spheron at low temperatures according to eqn. 1 (Sp = Spheron or Separon HEMA):

$$\text{Sp–OH} \xrightarrow[(H_2SO_4)]{SO_3} \text{Sp–O–SO}_3\text{H} \qquad (1)$$

In spite of the high chemical stability of Spheron, the method is rather drastic, as oleum has strong dehydration effects on organic compounds. The same reaction product may be obtained by using chlorosulphonic acid[5], either acting directly on Spheron or in the form of a solution in a suitable organic solvent:

$$\text{Sp–OH} + \text{ClSO}_3\text{H} \rightarrow \text{Sp–O–SO}_3\text{H} + \text{HCl} \qquad (2)$$

Instead of chlorosulphonic acid, ω-chloroalkylsulphonic acid may be used, which gives rise to sulphonic derivatives (b) according to eqn. 3:

TABLE I

EFFECT OF REACTION CONDITIONS ON THE CAPACITY OF THE CATION EXCHANGER OBTAINED IN THE ALKYLSULPHONATION OF SPHERON 1000 (20–40 μm)

To 10 g of Spheron swollen in 40 ml of water for 1 h, a 12.5 *M* solution of sodium hydroxide was added, followed after cooling to 20°C by a solution of sultone in 60 ml acetone. The solution was heated to the reaction temperature and maintained at this value, first by cooling and then by heating. Finally, the gel was removed by suction, washed with water, acetone, water and methanol and dried.

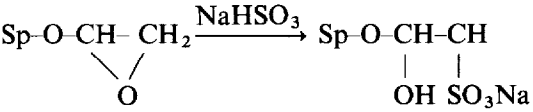
Amount of sultone added		*Amount of hydroxide added*		*Reaction time*	*Sulphur content by elemental analysis*	*Exchange capacity (mequiv./g)*	
g	*mmol*	*ml*	*mmol*	*(h)*	*(%)*	*Calc. from sulphur content*	*Determined titrimetrically (cf., Fig. 1)*
Effect of the amount of 1,3-propanesultone and alkali hydroxide at 30–°C							
12.2	100	28	350	1	1.35	0.42	0.42
24.4	200	36	450	1	2.30	0.72	0.64
47.2	350	48	600	1	3.50	1.09	1.08
61.0	500	60	750	1	4.98	1.56	1.50
164.4*	1200*	116	1450	1	5.61	1.75	1.79
61.0**	500**	120	1500	1	6.47	2.02	2.08
Effect of the reaction time in the modification by 1,3-propanesultone at 30°C							
61.0**	500**	80	1000	0.5	4.55	1.42	1.40
61.0**	500**	80	1000	1	5.31	1.66	1.65
61.0**	500**	80	1000	2	5.18	1.62	1.67

* Sultone dissolved in 100 ml acetone.

** Gel suspended in NaOH solution (alkaline swelling).

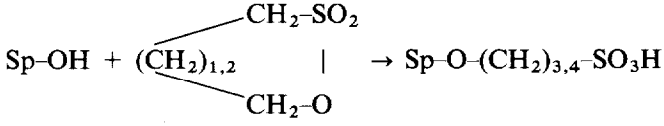
$$\text{Sp–OH} + \text{Cl(CH}_2\text{)}_2\text{SO}_3^- \xrightarrow[(\text{OH}^-)]{-\text{HCl}} \text{Sp–O–(CH}_2\text{)}_2\text{–SO}_3^- \qquad (3)$$

The fourth route leads also to type (b) from the glycidyl derivative of Spheron, the preparation of which proceeds according to eqn. 2a in ref. 4. The glycidyl derivative reacts with sodium bisulfate under mild conditions according to:



(4)

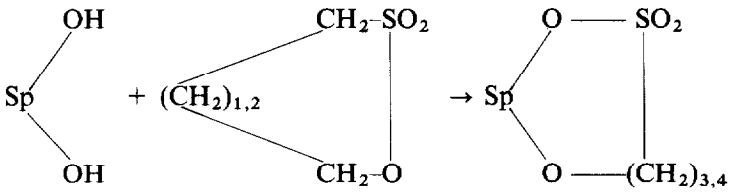
Strongly acidic cation-exchange derivatives of Spheron may readily be prepared on a technical scale using cyclic sultones[7]:



(5)

At first, propanesultone was used (SP derivatives are formed, Table II). However, as this compound is carcinogenic, butanesultone is now used (SB derivatives are formed, Table II).

Some ion exchangers prepared for chromatographic purposes are characterized analytically in Table II. For these derivatives and some similar ones, small differences were observed between the nominal capacities determined titrimetrically and those calculated from the sulphur content according to elemental analyses (Table II). Although these differences lie close to the limit of accuracy of the analytical methods, they are corroborated by the results of repeated experiments. When the ion exchangers were prepared by rapid reactions at normal temperatures, the capacity calculated from the sulphur content was usually slightly higher than the analytically determined value, *cf.*, Fig. 3. We explain this by the side reaction



(6)

leading to a non-ionogenic product which may appear in the products of reaction 5. In contrast when the reaction was carried out for an extended time in a strongly alkaline medium, *cf.*, Table I, and particularly at high temperature, *cf.*, Fig. 1, the analytically determined nominal capacity was somewhat higher than that calculated theoretically from the sulphur content. This was predominantly observed in the re-

TABLE II

ANALYTICAL CHARACTERISTICS OF SOME SULPHO-SPHERONS PREPARED IN THIS WORK

Data for unsubstituted Spheron 1000 are given in column 0 for the sake of comparison. For further data on all three Spherons 100, 300 and 1000 see ref. 1.

Property	*Unit*	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Starting Spheron		1000	1000	1000	1000	1000	1000	1000	1000	300	300
Type of substitution*		–	SP	SP	SP	SP	SP	SP	SP	SB	SB
Particle size	μm	20–40	20–40	20–40	20–40	20–40	20–40	20–40	20–40	40–63	40–63
Nominal capacity											
Calculated from S content	mequiv./g	0	0.43	0.62	1.15	1.68	1.73	1.60	1.49	1.66	2.08
Determined titrimetrically**	mequiv./g	0	0.42	0.63	1.09	1.72	1.66	1.56	1.63	1.64	2.00
Determined by column method***	mequiv./g	0.04	0.37	0.60	1.00	1.50	1.55	1.45		1.52	1.99
Elemental analysis											
C	%	55.47				45.42			45.97	46.21	44.92
H	%	7.57				7.37			7.18	7.61	7.81
S	%	0	1.38	1.99	3.67	3.58	5.52	4.82	4.78	5.37	6.67
Dry matter	%	96.69	96.10	95.20	93.00	88.74	90.90	91.57	87.60	85.76	84.05
Working volume§											
In 2 *M* NaOH	ml/g	3.80	3.7	3.3	3.4	3.2	3.0	3.4		3.2	3.3
In water	ml/g		3.6	3.3	3.4	3.2	3.3	3.4		3.2	3.3
In 2 *M* HCl	ml/g	3.75	3.6	3.2	3.4	3.1	3.0	3.3		3.1	3.2
Specific internal surface area§§	m^2/g	140	101	74	78	53	47	68	37	63	42
Capacity for serum albumin by											
static method (desorption)§§§	mg/g	12	244	293	382	346	340	346		146	160
dynamic method†	mg/ml	5.5		47	65	78		60			

* SP = Sulphopropyl; SB = sulphobutyl.

** Ion exchanger in hydrogen form stirred in excess of alkali hydroxide was back-titrated with acid using phenolphthalein.

*** Excess of alkali hydroxide titrated was squeezed with distilled water through a column of ion exchanger (H^+) and back-titrated[1].

§ Measured in the determination of nominal capacity by column method[1].

§§ Determined by one-point Kljačko–Gurvič method cited in refs. 1–4.

§§§ Protein bound to ion exchanger in the test-tube by batch method in the presence of excess of protein was released by increasing the ionic strength and determined photometrically in supernatant[2].

† Ion-cxchanger column was saturated with protein, washed with sorption buffer and the protein was released by desorption buffer of high ionic strength[3].

actions with butanesultone. We explain this finding by a partial hydrolysis of the otherwise very stable glycol methacrylate matrix which is an ester[5]. This reaction may occur under more drastic conditions at elevated temperatures:

$$\text{Sp–CO–O–(CH}_2\text{)}_2\text{–OH} \xrightarrow[(\text{OH}^-)]{\text{H}_2\text{O}} \text{Sp–COOH} + \text{HO(CH}_2\text{)}_2\text{OH} \qquad (7)$$

The reaction results in the conversion of the neutral copolymer into a carboxylic cation exchanger, and may thus raise the nominal cation-exchange capacity above that calculated on the basis of the introduced sulpho groups.

Static and dynamic capacities of cation exchangers prepared for serum albumin sorption

The optimal pH value for the static sorption of serum albumin* determined by the batch method[2] was investigated in the range pH 3–6 and is shown in Fig. 2.

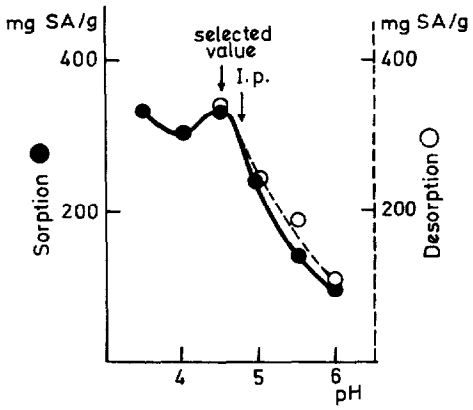


Fig. 2. Static capacities for serum albumin (SA) of Spheron sulphate 1000 (nominal capacity 1.72 mequiv./g) as a function of pH. The method of determination is described in ref. 2. The sorption buffer was 0.01 *M* NaOH + acetic acid, the desorption buffer was 0.1 *M* NaOH + acetic acid adjusted to 2 *M* NaCl (so that after 5 ml of supernatant had been removed[2] and 5 ml of the desorption buffer added, the final concentration was 1 *M* NaCl). In buffers of pH 3.5 and 4.0 the desorption could not be measured because of turbidity. I.p. = Isoelectric point of serum albumin.

This Figure is very similar to Fig. 2 in ref. 4 illustrating the sorption of serum albumin on Spheron phosphate. Also for sulpho-Spherons, the optimal pH for the sorption of serum albumin lay near the isoelectric point of this protein, and a comparison between these findings and the conventional view of protein sorption on ion exchangers has been discussed[4]. On the basis of the result in Fig. 2, pH 4.5 was chosen for the sorption of serum albumin on sulpho-Spheron. The same value was used for static desorption. The determined capacities of sulpho-Spherons for static desorption are summarized in Table II; some of them are used in the plot in Fig. 3.

* In the previous paper[4] the concentration of the sorption buffer was inadvertently given as 0.1 *M* both for the static and for the dynamic method. The correct concentration used for both methods over the whole series of studies[2–4] is 0.01 *M*.

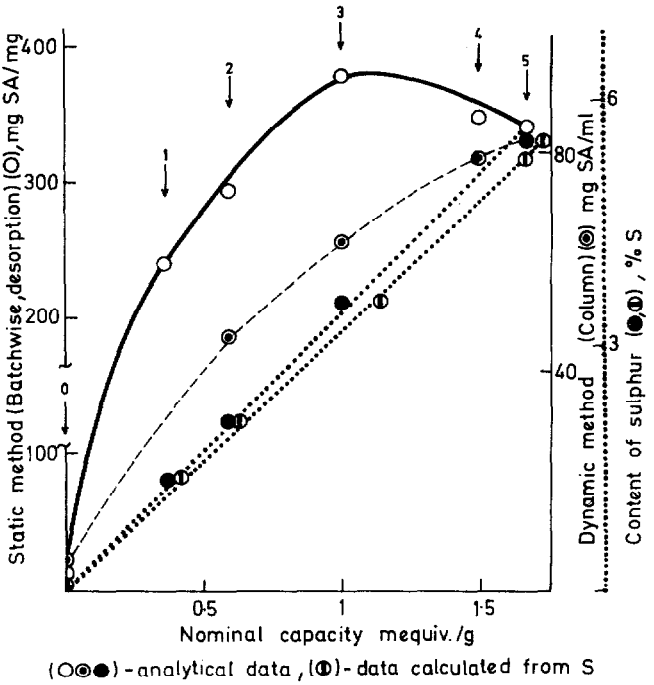


Fig. 3. Relationships between the nominal capacity for small ions (Na^+), analytically determined sulphur content, static capacity and dynamic capacity for serum albumin. The methods employed are described in the text. The ion-exchanger samples given at the arrows are denoted as in Table II.

The capacity for sorption of serum albumin was determined also by the dynamic method[3]. As the method has already been explained in detail and illustrated[3,4], no further details are given here. The pH value chosen for dynamic sorption was again 4.5, but pH 6.5 proved to be better for the desorption buffer used in the present study. The capacities for serum albumin determined by the dynamic method using a sulpho-Spheron column, 20 × 0.8 cm I.D., are given in Table II and used in the plot in Fig. 3.

Relationships between the sulphur content, nominal capacity for small ions (Na^+) and static and dynamic capacities for serum albumin of sulpho-Spheron are summarized in Fig. 3. An extensive discussion of these relationships for Spheron phosphates can be found in ref. 4, Fig. 3. For sulpho derivatives of Spheron, only the following comments are added. (a) The relationship between the sulphur content and nominal capacity for small ions is linear, similarly to the relationship between the nitrogen content and the anion-exchange capacity[2] and that between the phosphorus content and the cation-exchange capacity[4]. (b) The analytically determined nominal capacity of cation exchangers prepared by rapid reactions at normal temperatures is somewhat lower than the theoretically calculated one, as explained above. (c) The static capacity of such strongly acidic cation exchangers for serum albumin initially increases steeply with nominal capacity, but decreases slightly at higher capacities. This is in agreement with a similar finding for Spheron phosphates[4], except that in the present case it happens at lower capacities of *ca.* 1.5 mequiv./g. (d) The

dynamic capacity for serum albumin increases continuously with nominal capacity, but not linearly. These conclusions resemble those arrived at for Spheron phosphates and shown in Fig. 3 in ref. 4.

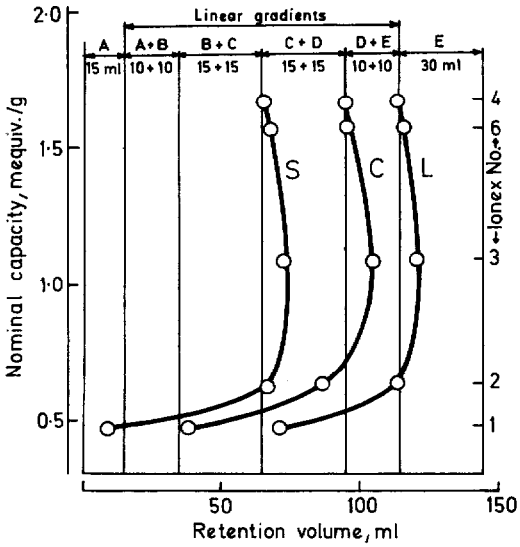


Fig. 4. Effect of the capacity of sulpho-Spheron on the retention volumes of components of an artificial mixture of serum albumin (S), chymotrypsinogen (C) and lysozyme (L) in gradient chromatography on columns of 20 × 0.8 cm I.D. using buffer solutions pH 4.3 prepared from alkali of the given final concentrations and citric acid: A, 0.01 *M* NaOH; B, 0.2 *M* NaOH; C, 0.6 *M* NaOH; D, buffer C + 1 *M* NaCl; E, buffer C + 2 *M* NaCl. Ion-exchangers denoted as in Table II.

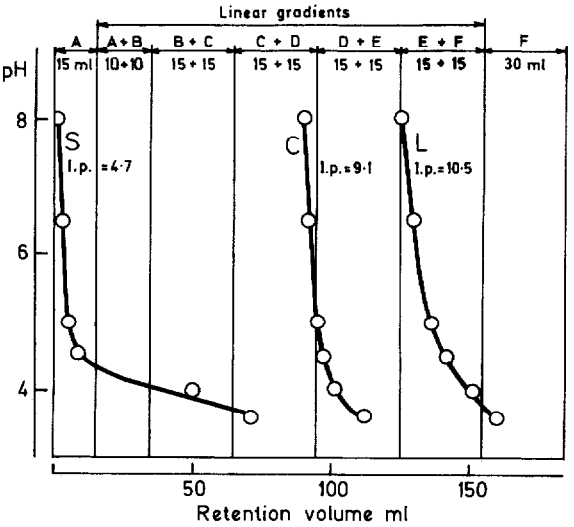


Fig. 5. Effect of pH on the separation of serum albumin (S), chymotrypsinogen (C) and lysozyme (L) by gradient elution on a column of sulphopropyl-Spheron 1000 having a capacity of 1.5 mequiv./g. The buffers were prepared from alkali of the given final concentrations and adjusted to the given pH values with citric acid (up to pH 5) and phosphoric acid (pH 6.5 and 8). The alkali concentrations were: A, 0.01 *M*; B, 0.05 *M*; C, 0.2 *M*; D, 0.5 *M*; E, buffer D + 1 *M* NaCl; F, unbuffered 2 *M* NaCl. I.p. = Isoelectric point.

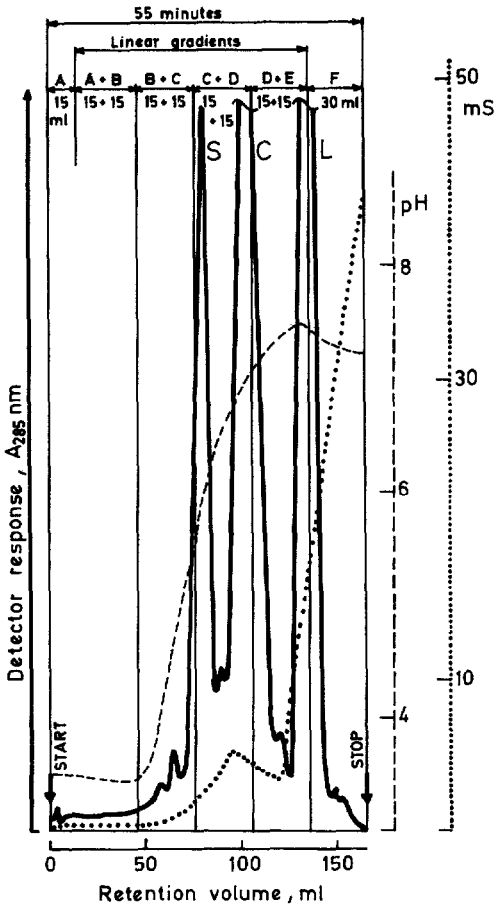


Fig. 6. Separation of 37 mg of a mixture of serum albumin (S), chymotrypsinogen (C) and lysozyme (L) on a column (20 × 0.8 cm I.D.) of sulphopropyl-Spheron (sample 4, Table II) by the use of anionic strength and pH gradient. Buffers used (citric acid for lower pH values, phosphoric acid at pH > 6.5): A, 0.01 *M* NaOH, pH 3.5; B, 0.05 *M* NaOH, pH 5.0; C, 0.2 *M* NaOH, pH 6.5; D, 0.5 *M* NaOH, pH 8.0; E, buffer D + 1 *M* NaCl; F, unbuffered 2 *M* NaCl. mS = Effluent conductivity in milliSiemens. Counterpressure: 4 atm. Flow-rate: 3 ml/min. Temperature: 13°C (column jacket cooled with tap-water).

Chromatographic experiments

Spheron sulphates and sulpho-Spherons have already been used for various chromatographic purposes (see studies cited in ref. 4). In ref. 5 Spheron sulphates were used; in other studies cited in ref. 4 the compounds employed were sulpho derivatives. In the present paper we comment on the effect of nominal capacity and pH on the separation of a synthetic mixture of serum albumin (S), chymotrypsinogen (C) and lysozyme (L) using sulpho-Spheron; an example of a separation of a natural mixture of egg proteins and a cellulolytic enzyme preparation is also given.

The effect of the capacity of an ion exchanger on the chromatographic analysis of an artificial mixture of proteins S, C and L is illustrated by Fig. 4, which resembles (although not markedly) the effect of capacity on the separation of the same proteins in the case of Spheron phosphates (Fig. 5 in ref. 4). In the case of the sulpho deriv-

atives the change in pH of the effluents was not as great as for the phosphates; also, the decrease in the retention volumes in the range of higher capacities was not so pronounced. The effect of pH on the separation of the same proteins can be seen in Fig. 5. A separation using a pH gradient is illustrated in Fig. 6. The pH gradient had no special advantage compared with experiments at constant pH.

Attempts were also made to separate a natural mixture of egg-white proteins, 20 mg, on a column of the sulphopropyl derivative of Spheron 1000, 20 × 0.8 cm I.D. at different pH values 3.5, 4.0, 4.5, 5.5 (in citrate buffers), 6.0 and 8.0 (in phosphate buffers). The optimum pH value was 5.0. At lower pH values the peak retention volumes became unnecessarily large, while at higher pH the quality of the chromatographic profiles was impaired. A separation at pH 5 is illustrated in Fig. 7.

Fig. 8 shows the possibility of using strongly acidic Spheron cation exchangers in the chromatography of active enzymes, without loss of activity, as has been demonstrated in the chromatography of glucose oxidase[5] and pectolytic enzymes[8,9].

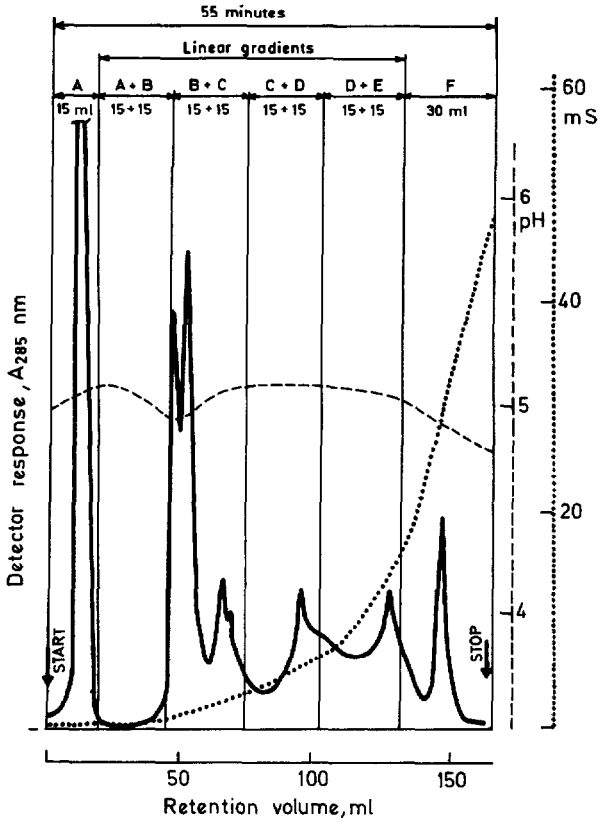


Fig. 7. Chromatography of 21 mg of egg-white protein on a column (20 × 0.8 cm I.D.) of sulphopropyl-Spheron, 20–40 μm (sample 4, Table II), using a system of linear gradients. All buffers had pH = 5.0 and were prepared from sodium hydroxide of the given concentrations and citric acid: A, 0.01 *M*; B, 0.05 *M*; C, 0.2 *M*; D, 0.05 *M*; E, buffer D + 1 *M* NaCl; F, unbuffered 2 *M* NaCl. mS = Effluent conductivity in milliSiemens. Flow-rate: 3 ml/min. Counterpressure: 6–8 atm. Temperature (maintained by flow of water through the column jacket): 14.5°C.

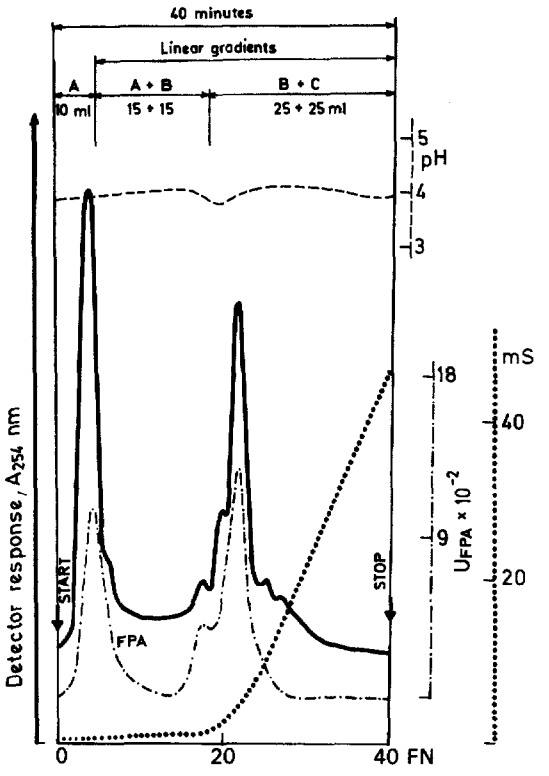


Fig. 8. Chromatography of 20 mg of the cellulolytic system from *Trichoderma viride-reesei* on a column (20 × 0.8 cm I.D.) of sulphopropyl-Spheron 1000 (20–40 μm), capacity 1.72 mequiv./g. The buffers used had pH 4.0 and were prepared from sodium hydroxide of the given concentrations and citric acid: A, 0.01 *M*; B, 0.05 *M*; C, 0.25 *M* (1 *M* in NaCl). · · · · = Effluent conductivity in milliSiemens; FPA = filterpaper activity determined in 0.8-ml aliquots of fractions by a procedure described in ref. 4; FN = number of fractions (volume 2.3 ml) taken in 60-sec intervals. Pressure: 5 atm, room temperature.

Catalytic properties of sulpho derivatives of glycol methacrylate gels

Similarly to Spheron phosphates[4], sulpho-Spheron may also be used as an "immobilized acid" in catalyzed reactions. As previously[4], the catalytic activity for the esterification of acetic acid with *n*-butanol was tested using model catalysts, except that the arrangement was a discontinuous one. According to the gas chromatographic results, the reaction mixture contained 85% *n*-butyl acetate, 11% acetic acid and 4% *n*-butanol. The exchange capacity of the ion exchanger decreased from 1.97 to 1.61 mequiv./g. The appearance of the ion exchanger remained virtually unchanged; only the beads that adhered to the walls of the flask turned yellow.

CONCLUSIONS

Both the sulphate and sulpho derivatives of Spheron may easily be prepared by using several technically feasible reactions; of these, the one in which cyclic sultones are used was examined in detail. The nominal capacities of the products are 1.5–2.0 mequiv./g, which is sufficient for the sorption of proteins and their chromatography. Similarly to phosphate derivatives, sulpho derivatives of Spheron be-

have as "immobilized acids" in attempts to use them as catalysts, but their general properties are not better than those of the phosphate catalysts described earlier[4].

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