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# ION-EXCHANGE DERIVATIVES OF SPHERON

## IV*. PHOSPHATE DERIVATIVES

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SUMMARY

Medium acidic phosphate derivatives of the hydroxyethyl methacrylate macroporous gels Spheron 300 and 1000 were prepared by several procedures. The resulting cation exchangers had nominal capacities of 0.20–4.08 mequiv./g and were characterized by their capacity for small ions, static and dynamic capacities for proteins, elemental analysis, operating volume and inner surface area. Some samples were titrated potentiometrically to the first and second dissociation degrees and the results discussed in terms of the phosphorus content. The relationships between the nominal capacity and phosphorus content, between nominal capacity and static capacity for proteins and between the static and the dynamic capacity for proteins were investigated and discussed. Chromatographic experiments on a mixture of serum albumin, chymotrypsinogen and lysozyme showed a dependence of retention volumes on the nominal capacity. Applications to the separations of egg-white proteins and of cellulolytic enzymes from a cultivation liquid of *Trichoderma viride-reesei* are described. Experiments with the cation exchanger Spheron 300 phosphate, used as an "immobilized acid" for catalysis (esterification of alcohol and inversion of saccharose), are also reported.

## INTRODUCTION

New types of ion exchangers based on the macroreticular poly(2-

* For Part I see ref. 2; for Part II, ref. 3 and for Part III, ref. 4.

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hydroxyethyl)methacrylate, trade-name Spheron (Lachema, Brno, Czechoslovakia) or Separon HEMA (Laboratory Instruments Works, Prague, Czechoslovakia), have been developed since 1975 for the rapid chromatography of biopolymers and their fragments. In our introductory work[1], these ion exchangers were described and examples presented of the chromatography of proteins, polypeptides, amino acids, nucleic acids, oligonucleotides and nucleotides. The hydrodynamic properties of these new packings for high-performance liquid chromatography (HPLC) of biopolymers were also discussed. We subsequently reported[2] the characterization of the polymer matrix, in particular its macrostructure and porosity, and described in detail[3] the medium-basic diethylaminoethyl (DEAE) derivatives of Spheron, including examples of their application and a method of determination of the static capacity for proteins. The most recent paper[4] in this series dealt with the weakly acidic carboxylic derivatives of Spheron and described a determination of the dynamic capacity for proteins.

These ion-exchange derivatives of Spheron and other derivatives not yet described in detail have found broad application in biochemistry for the separation of technical enzymes[5–7] and other proteins and their fragments[8], in the chromatography of mono- and oligosaccharides[9,10] including cellodextrins[11], mono- and oligonucleotides[12,13] and organic acids[14]. The rapid chromatographic analysis of enzymes and examples of the application of Spheron ion exchangers have been reviewed[15–17].

This paper describes medium-acidic phosphate Spheron cation exchangers and their application in the rapid chromatography of proteins and in catalysis (*cf.*, ref. 18). A part of the results have been presented in a preliminary form[19].

## EXPERIMENTAL

### *Materials*

Spheron 300 (beads 10–20 and 20–40 μm) and 1000 (beads 20–40 and 40–60 μm) were products of Lachema. Spheron 300 was extracted before ionogenic modifications, as described[2]. Phosphorylated derivatives of hydroxyethyl methacrylate gels of capacity 2.20 or 2.28 mequiv./g, applied in catalysis, were prepared according to ref. 20 from non-extracted Spheron 300 (particle size 300–400 μm, specific inner surface area 103 $m^2/g$).

Proteins employed for testing chromatographic properties were those used earlier[1–4]. Phosphoryl chloride, amines and solvents required for ionogenic modifications, *n*-butanol, acetic acid and saccharose used for testing the catalytic efficiency of phosphorylated derivatives and all inorganic compounds and chemicals for determination of cellulolytic activity were supplied by Lachema. Whatman No. 1 chromatographic paper was supplied by Whatman Labsales (Maidstone, Great Britain). The cultivation liquid *Trichoderma viride-reesei* was kindly provided by the Research Institute of Food Industry, Prague.

### *Preparation of ion exchangers for chromatographic application*

Spheron phosphates were prepared by reaction of phosphoryl chloride with Spheron as shown in Scheme I in Results and Discussion. One of the laboratory procedures is given in the legend of Fig. 1, which shows the effect of reaction time on the course of ionogenic substitution. Further modifications are described in Table I,

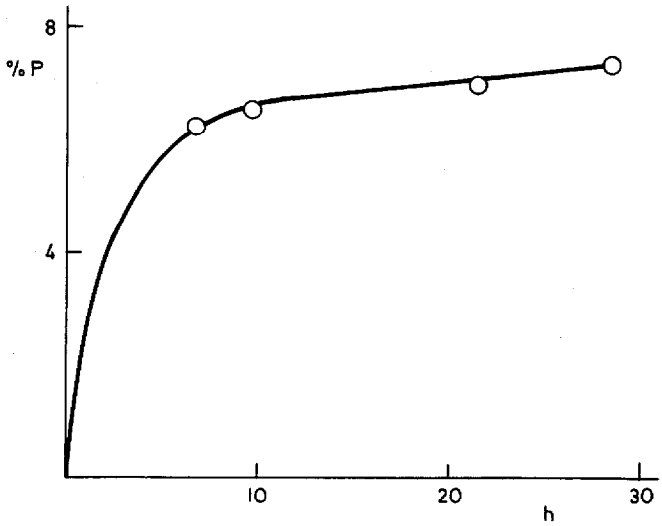



Fig. 1. Effect of reaction time on the ionogenic modification of Spheron. 2.5 g dry Spheron 300 (fraction of particle size 25 μm) dispersed in 25 ml ethyl acetate were phosphorylated, with stirring at −25°C, with 5 ml $POCl_3$ in the presence of excess of trimethylamine. The samples for analysis were washed with water and acetone, allowed to stand in 2 *M* NaOH for 2 h at laboratory temperature and further washed with water, ethanol, acetone and diethyl ether and dried.

which presents the effect of temperature, and in Table II, which gives characteristics of a series of ion exchangers with increasing nominal capacities.

In the preparation according to Scheme II (Results and Discussion), 10 g of Spheron were first transformed into a glycidyl derivative by alkaline swelling overnight in 50 ml of 50% potassium hydroxide at 10°C, followed by rapid filtration by suction and dispersion in 40 ml of epichlorohydrin in dioxan. After boiling under

TABLE I

INFLUENCE OF REACTION CONDITIONS ON CHLORINE AND PHOSPHORUS CONTENTS IN THE PREPARATION OF SPHERON PHOSPHATES

A 10-g amount of dried Spheron 300 (particle size 25 μm) was dispersed in 100 ml dry ethyl acetate at −30°C and, in the presence of 20 ml dry triethylamine, substituted stepwise by addition of 20 ml phosphoryl chloride dissolved in ethyl acetate for the given time. In the case of sample c, the temperature was increased to +25°C. The samples for analysis were treated as described in Fig. 1; they are not further characterized in Table III.

| *Sample* | *Reaction time (h)* | | *Elemental analysis (%)* | |
|---|---|---|---|---|
| | *At −25 to −30°C* | *At +25°C* | *Cl* | *P* |
| a | 1 | – | 0 | 2.68 |
| b | 3 | – | 0 | 3.76 |
| c | 3 | 15 | 3.20 | 5.27 |
| d | 6 | – | 0.12 | 7.25 |
| e | 7* | – | 0.19 | 6.95 |

* Pyridine was used instead of triethylamine and activated carbon was added after addition of $POCl_3$.

TABLE II

PREPARATION OF SPHERON PHOSPHATES OF INCREASING NOMINAL CAPACITY

Spheron 1000 (particle size 20–40 μm) was dispersed in chloroform, mixed with phosphoryl chloride at 0–5°C and pyridine dissolved in chloroform was added dropwise. After complete addition of pyridine, the mixture was stirred for another 2 h at the given temperature, filtered, washed and then subjected to hydrolysis in sodium bicarbonate solution. The resulting ion exchanger was cycled.

| *Sample No.** | *Phosphoryl chloride (mmol/g of gel)* | *Pyridine (mmol/g of gel)* | *Nominal capacity*** *(mequiv./g)* |
|---|---|---|---|
| 7 | 1.09 | 1.2 | 0.29 |
| 8 | 2.18 | 2.4 | 0.45 |
| 9 | 3.27 | 3.6 | 0.73 |
| 10 | 4.36 | 4.8 | 1.24 |
| 11 | 6.53 | 7.2 | 2.34 |
| 12 | 7.85 | 8.6 | 3.14 |
| 13 | 12.00 | 13.2 | 4.18 |

* Numbering as in Table III.

** In contrast to the data in Table III, the values were read from titration curves.

reflux for 3 h the required derivative was collected on fritted glass filter and washed three times with acetone, once with water, three times with acetone and three times with diethyl ether. This glycidyl-Spheron 300 (0.3 g) was mixed with 2 g sodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and 3 ml water and heated in a sealed tube to 98°C for 90 h. The product was refined and cycled by stepwise washing with water, 2 *M* hydrochloric acid, water, ethanol, acetone and diethyl ether and dried under vacuum.

We also tried to prepare Spheron phosphate according to Scheme III (Results and Discussion): 0.5 g Spheron 100 and 2 g sodium hydrogen phosphate were heated with 1.5 ml water in a narrowed-neck tube which was placed in a heating block at 175°C as long as water distilled off. After cooling a second portion (1.5 ml) of water was added and the process was repeated 10–15 times over 7 h. The product was washed with water, 0.5 *M* hydrochloric acid, water, methanol and acetone and dried.

*Chromatographic methods and evaluation of fractions*

The procedures were described earlier[1–4] (determination of static capacity, see Fig. 1[3]; determination of dynamic capactity, see Fig. 4[4]), as were the chromatographic instrumentation, packing of columns, regeneration (2 *M* sodium chloride), cycling (2 *M* sodium hydroxide, 2 *M* hydrochloric acid) and equilibration of Spheron ion exchangers. The effluent from chromatographic columns was continuously evaluated by measuring its absorbance at 285 and 254 nm; the conductivity and pH of collected fractions were also determined. The activity of cellulolytic enzymes (so called filter-paper activity) was ascertained for 100-μl aliquots of collected fractions according to Mandels and co-workers[21,22] using squares (1 × 1 cm) of Whatman No. 1 paper and incubation in 1 ml 0.05 *M* citrate buffer (pH 4.7) for 3–14 h at 40°C; 200 μl of the supernatant were then used for the determination of reducing sugars according to Somogyi[23] and Nelson[24]. For details see the disertation of Hostomská[25].

*Measurement of the catalytic activity of Spheron phosphates*

*n*-Butanol was continuously esterified in a vertical glass cylinder (100 × 3 cm I.D.) furnished with a thermostating jacket and heated by circulation of heating oil. A thermometer probe (thermocouple) was placed in the column axis. The column consisted of three sections: the upper inlet section contained a 19-cm layer or a preheating packing of porous ceramics followed by a 2-cm layer of glass wool; the central section contained a 56-cm layer of ion-exchange catalyst (Spheron phosphate, 2.28 mequiv./g) and a 2-cm layer of glass wool; the lower discharge section consisted of 19 cm of ceramic packing resting on a 2-cm layer of glass wool. The column was connected through a pump and a flow-controlling valve to a reservoir of processed liquid. The outlet of the column led to a separation vessel.

Continuous inversion of saccharose was carried out in a similar column (110 × 1.5 cm I.D.); the height of the catalyst section (Spheron phosphate, 2.20 mequiv./g) was 51 cm and the volume of this section was 90 ml.

## RESULTS AND DISCUSSION

*The phosphorylated derivatives of Spheron*

Medium-acidic cation exchangers of the Spheron phosphate type are best prepared by the reaction of phosphoryl chloride with Spheron[1,2] or Separon HEMA[26] (Sph–OH), dispersed in an organic solvent in the presence of a base, followed by hydrolysis:

$$\text{Sph–OH} + \text{ClPOCl}_2 \xrightarrow{-\text{HCl}} \text{Sph–O–POCl}_2 \xrightarrow{+\text{H}_2\text{O}} \text{Sph–OPO(OH)}_2 \qquad \text{(I)}$$

Analytical characteristics of the derivatives prepared by this method are listed in Table III.

Other methods of preparation were also studied, *e.g.* via the glycidyl derivative (prepared by use of epichlorohydrin, Scheme IIa) and the reaction with sodium phosphate (Scheme IIb):

$$\text{Sph–OH} + \text{ClCH}_2\text{–CH(O)–CH}_2 \xrightarrow{\text{KOH}} \text{Sph–O–CH}_2\text{–CH(O)–CH}_2 \qquad \text{(IIa)}$$

$$\text{Sph–O–CH}_2\text{–CH(O)–CH}_2 + \text{Na}_2\text{HPO}_4 \rightarrow \text{Sph–O–CH}_2\text{–CH(OH)–CH}_2\text{–O–PO(ONa)}_2 \qquad \text{(IIb)}$$

The attempted preparation by heating Spheron with sodium dihydrogen phosphate is illustrated by Scheme III:

$$\text{Sph–OH} + \text{HOPO(ONa)}_2 \xrightarrow{-\text{H}_2\text{O}} \text{Sph–O–PO(ONa)}_2 \qquad \text{(III)}$$

Other procedures, not described, were also tried. However, the route in Scheme I proved most suitable. It is of great importance in this case that the reaction product is the pure ion exchanger with a nominal capacity as high as possible, and it should not

TABLE III

ANALYTICAL CHARACTERISTICS OF THE PREPARED SPHERON PHOSPHATES

Data for the initial non-substituted Spherons are given for comparison, *cf.*, also refs. 2–4. Properties of the initial Spherons 300 and 1000 are given in ref. 2. Both types differ in pore size and in exclusion limit; the latter for Spheron 300 (after extraction) is 300,000–500,000 Daltons and for Spheron 1000 is $10^6$ Daltons.

| *Property* | *Unit* | *Non-substituted Spheron* | | *Cation exchangers* | |
|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* |
| Starting type | | 300 | 1000 | 300* | 300 |
| Particle size | μm | 20–40 | 20–40 | | 40–60 |
| Nominal capacity | | | | | |
| calculated (bifunctional groups) | mequiv./g | – | – | 4.21 | 2.25 |
| determined** | mequiv./g | 0.03 | 0.04 | 3.00 | 1.50 |
| Elemental analysis | | | | | |
| C | % | 55.68 | 55.47 | 46.25 | 51.33 |
| H | % | 7.56 | 7.57 | 6.26 | 6.47 |
| P | % | 0 | 0 | 6.52 | 3.49 |
| Cl | % | 0 | 0 | 1.42 | 0 |
| Ashes | % | 0 | 0 | | 0 |
| Dry substances | % | 100 | 96.69 | | 97.66 |
| Working volume** | | | | | |
| in 2 *M* NaOH | ml/g | 3.95*** | 3.80 | 2.7 | 4.15 |
| in 2 *M* HCl | ml/g | | 3.75 | 2.5 | 4.10 |
| Specific inner surface area | | | | | |
| by Klyaschko Gurwich method** | $m^2/g$ | 42–62 | 139–155 | 55 | 102 |
| Capacity for serum albumin (desorption)** | | | | | |
| by static method | mg/g | 2–3 | 12 | | |
| by dynamic method | mg/g | | 5.5 | | |

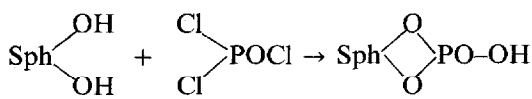
* Spheron was subsequently twice phosphorylated and the preparation contained 0.16% N.

** The methods are described in refs. 2–4.

*** Valid for distilled water.

contain any chlorine which may result in undesirable reactions. As is seen from the analyses in Table III, our procedures for ion exchanger preparation meet these requirements quite well.

One problem in the preparation of medium-acidic Spheron phosphate cation exchangers was that most samples exhibited a higher content of phosphorus than that corresponding to the capacity for small ions[2] as determined by titration of the presumed bifunctional group (*cf.*, Tables III and IV). This can be explained by a partial reaction of phosphoryl chloride with two adjacent hydroxyl groups on the surface of Spheron according to Scheme IV:




(IV)

| *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* |
|---|---|---|---|---|---|---|---|---|
| 300 | 300 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| 40–60 | 40–60 | 20–40 | 20–40 | 20–40 | 20–40 | 20–40 | 20–40 | 20–40 |
| 3.54 | 4.22 | 0.17 | 0.30 | 0.70 | 1.50 | 2.94 | 3.38 | 5.04 |
| 2.60 | 3.40 | 0.20 | 0.37 | 0.65 | 1.21 | 2.21 | 3.04 | 4.08 |
| 47.62 | 46.66 | 55.04 | 55.06 | 53.35 | 50.80 | 48.31 | 46.06 | 42.00 |
| 6.64 | 7.02 | 7.37 | 7.57 | 7.46 | 7.13 | 6.93 | 6.93 | 6.90 |
| 5.48 | 6.54 | 0.18 | 0.47 | 1.08 | 2.32 | 4.56 | 5.24 | 7.81 |
| 0.34 | 0.36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.37 | 0.28 | 0 | 0 | 0 | 0 | 0.38 | 0.19 | 0.31 |
| 97.38 | 97.41 | 96.43 | 96.57 | 97.53 | 97.05 | 96.45 | 95.73 | 95.60 |
| 4.0 | 3.8 | 3.75 | 3.68 | 3.50 | 3.50 | 3.40 | 3.25 | 3.10 |
| 3.65 | 3.65 | 3.70 | 3.65 | 3.45 | 3.45 | 3.30 | 2.90 | 2.80 |
| 100 | 100 | 73 | 88 | 96 | 92 | 99 | 94 | 92 |
| | | 108 | 164 | 178 | 216 | 220 | 224 | 196 |
| | | | 14.5 | | 21.8 | 23.7 | | 27.5 |

The partial formation of polyphosphate chains in the anhydrous medium can also be considered, as in Scheme V:

$$\text{Sph–OH} + 2\ \text{ClPOCl}_2 \rightarrow \text{Sph–O–}\underset{\underset{\text{OH}}{|}}{\text{PO}}\text{–O–PO(OH)}_2 \qquad \text{(V)}$$

As follows from Schemes IV and V, the exclusive occurrence of the first reaction (IV) would cause a more significant lowering of the nominal capacity, *i.e.*, from a ratio $2H^+$:P for Scheme I to $H^+$:P for Scheme IV. On the other hand, the exclusive reaction of type V would decrease the capacity only to the ratio $3H^+$:2P.

The potentiometric titration of samples of Spheron phosphates carried out to the first degree (Table IV) always agreed with the content of phosphorus found by elemental analysis. On the contrary, potentiometric titration to the second degree (Table IV) showed a clearly lower value than that corresponding to the content of

TABLE IV

PHOSPHORUS CONTENTS AND CALCULATED AND MEASURED CATION-EXCHANGE CAPACITIES OF SPHERON PHOSPHATES FOR BOTH DISSOCIATION DEGREES

Numbers of samples 4–6 as in Table III. Samples f and g were prepared in small amounts and are not characterized in Table III.

| *Sample No.* | *Content of P* | | *Nominal capacity (mequiv./g)* | | | |
|---|---|---|---|---|---|---|
| | *%* | *mmol/g* | *Calculated from P content for the bifunctional group* | *Experimentally determined* | | |
| | | | | *Potentiometrically, read from titration curves to the degree* | | *In effluent after passing titrated hydroxide through the column ($H^+$) and reverse titration using indicator according to ref. 2* |
| | | | | *I* | *II* | |
| f | 0.90 | 0.29 | 0.58 | 0.30 | 0.48 | |
| 4 | 3.49 | 1.13 | 2.25 | 1.02 | 1.62 | 1.50 |
| 5 | 5.48 | 1.77 | 3.54 | 1.75 | 2.86 | 2.60 |
| 6 | 6.54 | 2.11 | 4.22 | 2.18 | 3.61 | 3.40 |
| g | 7.10 | 2.29 | 4.58 | 2.29 | 3.66 | |

phosphorus. The nominal capacities, determined by filtration of an excess of titrated hydroxide through the ion-exchanger column according to ref. 2, exhibited even lower values (Table IV). This problem has not yet been solved fully, but it can be supposed that some rôle is played by those dissociable protons which would be potentiometrically titrated in the second degree, but which are eliminated by the formation of phosphoric diesters (IV) or polyphosphates (V).

*Static and dynamic capacity of Spheron phosphates for sorption of serum albumin*

The static sorption capacity of Spheron cation exchangers for proteins, as determined in slowly rotating sealed test-tubes[3], was first measured in several buffer solutions (pH 3–6) to find the optimal pH (see Fig. 2). The strongly acidic buffers prevented accurate measurements due to the formation of a turbidity in the solutions, in particular during desorption; on the other hand, the sorption decreased in the alkaline pH region. However, a shoulder was found in the dependence of static sorption on pH, for this ion exchanger, and the optimal pH, which enabled measurement of sorption and desorption, was at pH 5, *i.e.*, near the isoelectric point of serum albumin (pH 4.7). This is in contradiction to the usual belief that proteins are best sorbed on ion exchangers at a pH which differs by at least one pH unit from the isoelectric point (*cf.*, ref. 27).

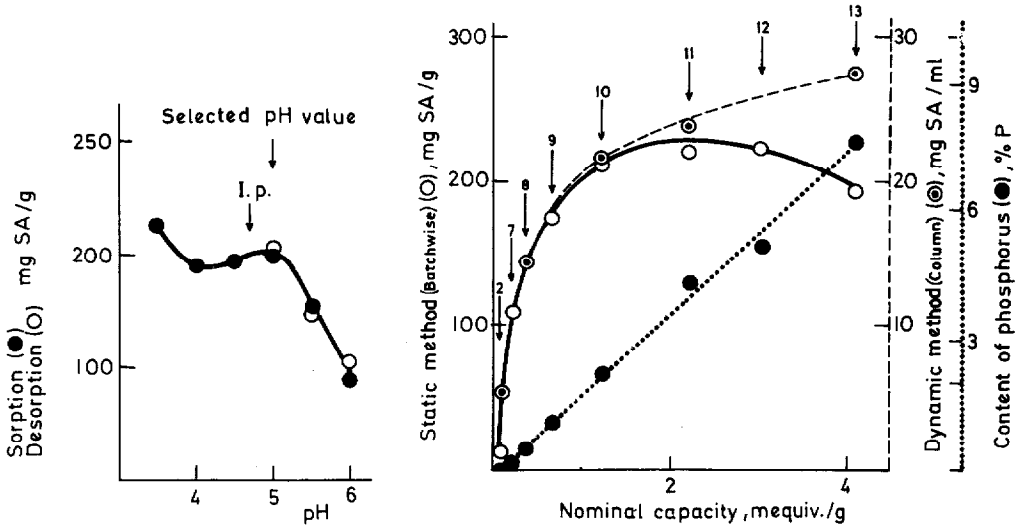



Fig. 2. Search for the optimal pH for the sorption of serum albumin on Spheron phosphate 300 of nominal capacity 3.4 mequiv./g (sample 6 in Table III), equilibrated with 0.1 *M* sodium acetate at various pH values. The sorption (and desorption in the same buffers adjusted to 1 *M* with NaCl) was studied under conditions described for the determination of static capacity by the batch method[3]. I.p. = Isoelectric point of serum albumin.

Fig. 3. Relationship between the nominal capacity of Spheron phosphates and the phosphorus content and between the static and dynamic capacities for sorption of serum albumin and the nominal capacity for small ions ($Na^+$). The static capacity was determined according to ref. 3 by a batch method in the presence of excess of protein in a sorption buffer (0.1 *M* sodium acetate, pH 5) as long as the equilibrium was established, followed by desorption with the same buffer enriched with 1 *M* NaCl. The dynamic capacity was determined at flow through a column according to ref. 4, under conditions given in Fig. 4. The sample numbers are as in Table III.

We have found (*cf.*, Fig. 4 in ref. 3), that the static sorption on DEAE-Spheron anion exchanger follows a steep convex curve depending on its nitrogen content, whereas the capacity for small ions, *e.g.*, $Cl^-$, has a linear dependence. We have also described the similar steeply convex dependence of the static capacity for serum albumin on the nominal capacity of carboxylic cation exchangers (CM-Spheron and Spheron C; *cf.*, Fig. 7 in ref. 4), and explained this feature on the basis of the multiple sorption of proteins on these ion exchangers under the conditions used to determine the static capacity[3,4]. In the present study of medium-acidic cation exchangers of the Spheron phosphate type, we have confirmed the earlier findings, as follows from Fig. 3. Thus the nominal capacity for small ions ($Na^+$) is roughly linearly dependent on the phosphorus content. The static capacity for serum albumin again steeply increases at low nominal capacities for small ions up to values of 2–3 mequiv./g. From a comparison of the results obtained with the three mentioned types of ion exchangers, it is obvious that the relationships between the capacity for small ions and for proteins follow the same general rule, thus confirming and generalizing the above hypothesis[3,4]. However, at even higher values of nominal capactiy a distinct drop in the static capacity occurs. A weak indication of this phenomenon was also found in Fig. 7 of ref. 4 where the static capacity of sample 4 is somewhat lower than that of sample 3. At that time[4], we ascribed this to possible scatter due to experimental errors. The phenomenon is much more pronounced in the present Fig. 3, because the phosphate ion exchangers have higher nominal capacities. Further experiments will be required to elucidate completely the reasons for this behaviour.

The measurement of the dynamic capacity for serum albumin (SA) is illustrated in Fig. 4 and the values found are given in Table III. The relationship between the static and dynamic capacities (Fig. 3) is surprisingly different from the expected nearly linear relationship between dynamic capacity and nominal capacity, found earlier for proteins on the carboxylic derivatives of Spheron (Fig. 7 in ref. 4). The dynamic capacity first follows a convex dependence similar to the static capacity, but with much lower values (these are 3–4 times higher after recalculation of volume data in mg SA/ml to weight data in mg SA/g), and increases almost linearly only above the nominal capacity of about 1.2 mequiv./g. It may be concluded that the sorption is controlled by at least two different mechanisms, depending on the nominal capacity. The bifunctional phosphate groups obviously adsorb proteins at low capacities relatively more intensively than do monofunctional groups, so that the course of the dynamic sorption at low nominal capacities may be similar to that of the static sorption.

*Chromatographic experiments*

Spheron phosphates are well suited as medium-acidic ion exchangers for the rapid chromatography of proteins. They have relatively high nominal capacities and suitable flow-through properties. The examples given below show that for chromatographic purposes it is not necessary further to increase their capacity. Examples of application of Spheron phosphates to the chromatography of proteins have been presented in previous papers[1,6–8,15]. For this reason, we now describe only an example illustrating the effect of nominal capacity on the separation of a synthetic mixture of proteins and two examples of the separation of protein mixtures.

*Separation of a synthetic mixture of human serum albumin (S), bovine chymo-*

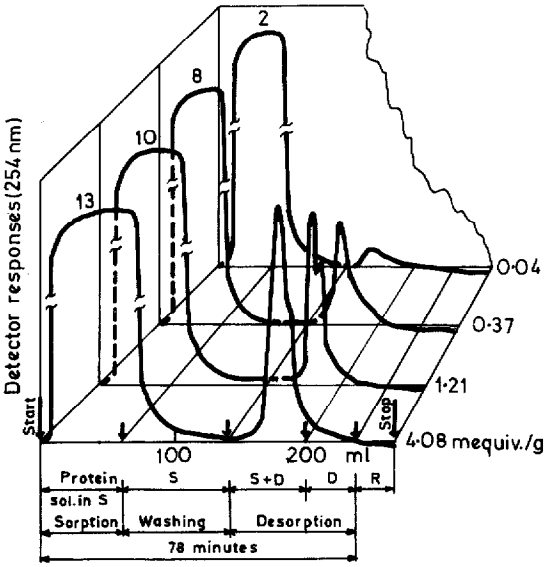



Fig. 4. Determination of the dynamic capacity of Spheron phosphates for serum albumin by the method of ref. 4 in a chromatographic column of 0.8 cm I.D. containing 10 ml of ion exchanger (height of packing about 20 cm). The numbers of the individual chromatographic profiles correspond to the ion-exchanger samples in Table III; their nominal capacities are given on the right. A 5% solution of serum albumin in the sorption buffer (S) was pumped through the column which had previously been equilibrated with the buffer S. After saturation with protein, the column was washed with buffer S to remove serum albumin which was not ionically bonded, and then the desorption was carried out. The flow-rate was 3 ml/min during the whole experiment; 20°C; pressure drop 0.4–0.5 MPa. S = Sorption buffer, 0.1 *M* sodium acetate, pH 5; D = desorption buffer of the same composition, enriched with 2 *M* NaCl; S + D = region of linear gradient; R = regeneration with non-buffered solution of 2 *M* NaCl. The desorption effluent was collected, its volume measured and analyzed spectrophotometrically for the content of eluted serum albumin, which gave the dynamic capacity (mg SA/ml) of the ion-exchanger bed.

*trypsinogen (C) and chicken lysozyme (L).* Elution was first attempted on a column (0.8 × 20 cm) containing Spheron phosphate of high capacity (4.08 mequiv./g) equilibrated with the first buffer, using a series of linear-gradients of buffers with simultaneously increasing pH and ionic strength in the region from 0.05 *M* sodium formate (pH 3.5) to 1 *M* sodium acetate (pH 8); in the last gradient 1 *M* sodium acetate (pH 8) was used with the same buffer enriched to 1 *M* sodium chloride. Because serum albumin tended to be eluted together with chymotrypsinogen under these conditions, a series of experiments were carried out with a gradient of ionic strength only, at pH 4, 5, 5.5, 6, 6.5 and 7.5 respectively. The results obtained resembled closely those from similar experiments carried out previously with the carboxylic cation exchanger (Fig. 10 of ref. 4); only the pH value at which S and C start to separate was different. Only two peaks (S and C were combined) were observed in both series of experiments at low pH, whereas the proteins S, C and L were separated at higher pH, but S and C were separated discontinuously (*cf.*, the discussion in ref. 4 relating to Fig. 10). The experiment with CM-Spheron[4] mentioned above showed a threshold pH value of 6.25, whereas for Spheron phosphate this appears at pH 5.5. Because this interesting chromatographic behaviour occurred with two different Spheron ion exchangers, we presume a similar interaction of both proteins. The value of pH 6.5 was chosen as optimum for further separations.

The following experiments were carried out to investigate the effect of the degree of ionogenic substitution on the separation of the mixture of proteins S, C and L under optimal conditions, starting with the unsubstituted Spheron (low nominal cation-exchanging capacity of 0.04 mequiv./g due to a trace of irremoved carboxyls and proceeding to the strongly modified Spheron phosphate (4.08 mequiv./g). In contrast to the earlier experiments with DEAE-Spheron[3] and CM-Spheron[4] where the ion exchanger with highest capacity proved most efficient, the present experiments revealed that the mixture of proteins is well separated on the ion exchanger with lower nominal capacities (*cf.*, Fig. 5). Of interest was the shifts in position of the peaks of the basic lysozyme L with decreasing capacity; these first appeared at higher retention volumes and then rapidly dropped to the hold-up volume, in contrast to an expected continuous decrease with decreasing capacity. This behaviour can be explained on the basis of an antagonistic effect of the spontaneously decreasing pH with the increasing nominal capacity upon the retention of absorbed proteins. The same tendency, although not so pronounced, can be seen with the other proteins C and S. It is obvious that Spheron phosphate with bifunctional groups differs from DEAE-Spheron and CM-Spheron not only in the dependences of the static and dynamic capacities for proteins

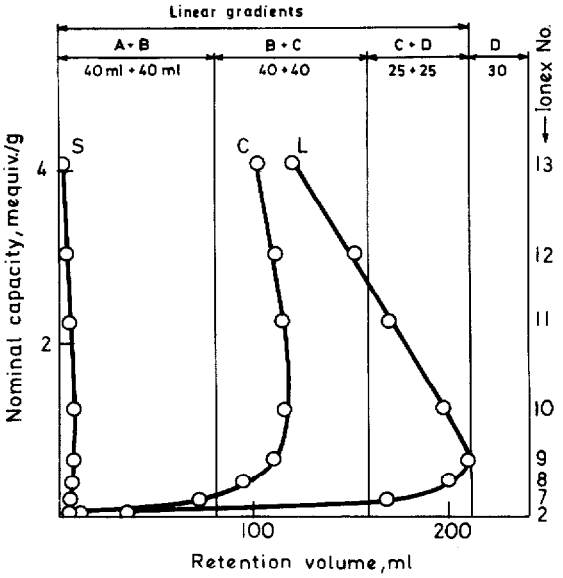



Fig. 5. Dependence of separation of 20.5-mg mixture of serum albumin S (10 mg), chymotrypsinogen C (7 mg) and lysozyme L (3.5 mg) on the nominal capacity of Spheron phosphate of particle size 20–40 μm. The column dimensions were in all cases 20 × 0.8 cm I.D. and the mixture of proteins was applied in 0.3 ml of the first buffer, which was also used for equilibration of the ion exchangers. The numbers of the ion exchangers are as in Table III. The points indicate the positions of chromatographic peak maxima. All buffers were prepared from ammonium hydroxide of the following concentrations adjusted by acetic acid to pH 6.5: A, 0.05 *M*; B, 0.3 *M*; C, 1 *M*; D, non-buffered 2 *M* NaCl. The flow-rate was 3 ml/min; pressure drop 0.5–0.9 MPa; ambient temperature. The pH during elution with the linear gradients was constant at 6.5 for unsubstituted Spheron (No. 2, Table III) but decreased for cation exchangers in the region between the first and second gradient, becoming more pronounced with increasing nominal capacity (from pH 6.4 to 6.2, whereas a slow but incomplete return to the original pH value took place with further elution at the end of gradients).

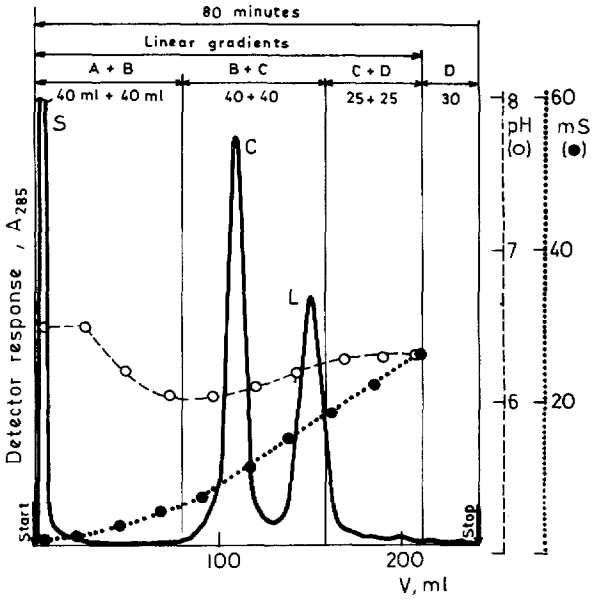



Fig. 6. Separation of serum albumin (S), chymotrypsinogen (C) and lysozyme (L) on Spheron phosphate 1000 of capacity 3.04 mequiv./g (preparation 12, Table III). For chromatographic conditions see Fig. 5. The volume of effluent, $V$, is shown on the horizontal axis.

on the nominal capacity for small ions, but also in the chromatographic retention. An example of the chromatographic profile of the separation of three proteins is shown in Fig. 6.

The egg-white proteins were chosen as an example of a natural mixture of proteins, as previously for CM-Spheron (Figs. 8 and 9 in ref. 4). Experiments only with a gradient of ionic strength in ammonium acetate buffers at pH 4, 7 and 9 failed. However, simultaneously increasing ionic strength and pH from 4.5–5.5 to 8–9 proved suitable for the separation of these proteins. In contrast to the behaviour shown in Fig. 5, the ion exchangers with the highest nominal capacities gave the best separation in these experiments, as with other types of Spheron ion exchangers. Fig. 7 shows the chromatogram obtained using Spheron phosphate of high capacity (4.08 mequiv./g). A reasonable separation was even obtained with ion exchangers of lower capacity down to 2.27 mequiv./g. Still lower capacities led to an unsatisfactory separation; the peaks broadened and the unsubstituted starting Spheron 1000 with gradients of ionic strength and pH did not separate the egg proteins.

An example of enzyme separation on Spheron phosphate is shown in Fig. 8 for the chromatography of cellulolytic enzymes from a cultivation liquid *Trichoderma viride-reesei*. The so-called "filter-paper activity" was only tested in this experiment, and was found to be fairly retained, although the ion exchanger (No. 3, Table III) contained small amounts of bonded chlorine. Other types of enzymes from the cellulolytic system of this organism retained full enzyme activity during their separation on Spheron phosphates. The separation of cellulolytic enzymes on Spheron ion exchangers proved very suitable[25] and will be reported in a separate paper[28].

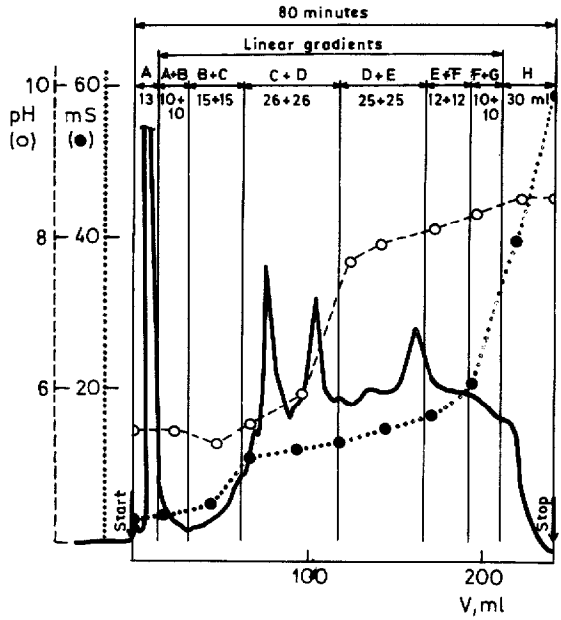



Fig. 7. Chromatography of 20-mg egg-white protein preparation on a column (20 × 0.8 cm I.D.) packed with Spheron phosphate 1000 of capacity 4.08 mequiv./g and particle size 20–40 μm (preparation 13, Table III). Buffers A and B were prepared from 0.01 *M* and 0.2 *M* NaOH adjusted to pH 5.5 and 6.5, respectively, with acetic acid. Buffers C (0.4 *M*), D (0.6 *M*), E (0.6 *M*), F (0.6 *M*) and G (0.6 *M*) were prepared from Tris adjusted with acetic acid to pH 7.5, 8.0, 8.5, 9.0 and 9.0 and enriched with 0.1 *M*, 0.2 *M*, 0.4 *M*, 1.0 *M* and 2.0 *M* NaCl. The non-buffered regeneration solution (H) was 2 *M* NaCl. Flow-rate 3 ml/min, 25°C, pressure drop 0.6 MPa. The fractions were collected every 90 sec.

*Catalytic properties of Spheron phosphates*

Cation exchangers in the hydrogen form are "immobilized acids" and may serve as catalysts in reactions which are otherwise catalyzed by hydrogen ions. We chose two types of such reactions to study the catalytic properties of Spheron phosphates: (a) continuous esterification of acetic acid with *n*-butanol and (b) the continuous inversion of saccharose.

The catalyst of initial nominal capacity 2.28 mequiv./g was used in the experiments on esterification of acetic acid with *n*-butanol. Best results (90 % conversion of acid) were achieved at the acid:alcohol molar ratio of 1:1.2, 116°C and flow-rate 20 ml/h. When the highest conversion degree was attained, the product formed on cooling was a two-phase system with the mixture of ester and alcohol in the upper layer. At lower degrees of conversion the product formed one phase. The degree of conversion of acid (but not the relative content of ester in the resulting mixture) rose with increasing proportion of alcohol (from 1:1 to 1:2), but decreased with decreasing temperature (from 110 to 60°C) or with increasing flow through. At flows higher by one order of magnitude (about 200 ml/h) the degree of conversion is very low. Traces of phosphorus were observed in the reaction products (*e.g.*, $2.5 \times 10^{-3}$ %), slightly increased in amount with temperature at 60–70°C and rapidly increased above 85–90°C. A conversion degree of 74 % was obtained at 79°C, the acid:alcohol molar ratio

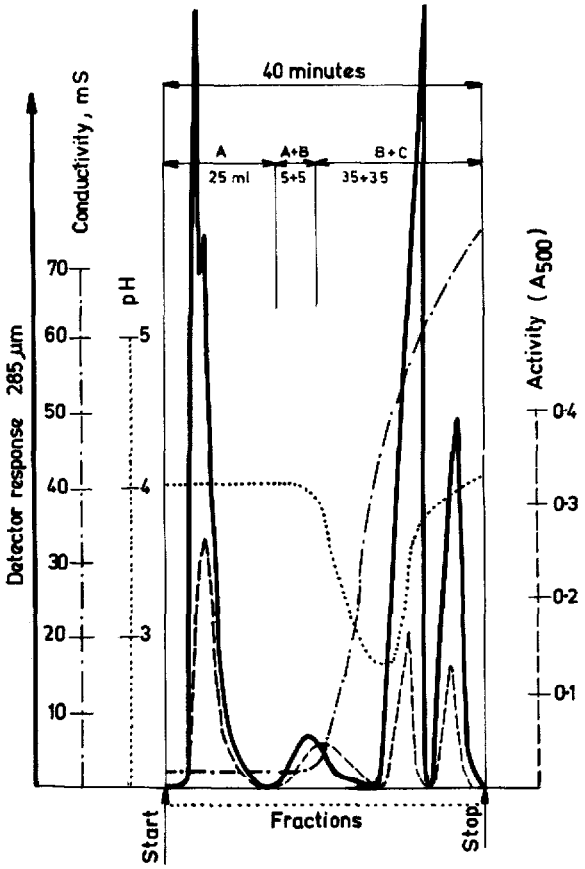



Fig. 8. Medium-pressure chromatography of a cellulolytic preparation from the cultivation liquid *Trichoderma viride-reesei* on a column (20 × 0.8 cm I.D.) with Spheron phosphate 300 of capacity 3.0 mequiv./g (sample 3, Table III). Buffers: A, 0.05 *M* NaOH adjusted with acetic acid to pH 4; B, the same buffer enriched to 1 *M* NaCl; C, the same buffer enriched to 3 *M* NaCl. Flow-rate 2.4 ml/min, 20°C. Fractions of volume 4.8 ml were collected in 2-min intervals: pressure drop 0.5 MPa. A method for measurement of cellulolytic activity by the analysis of reducing sugars is described in the text.

being 1:2 and the flow through 67 ml/h. The long-term behaviour of the column was tested, following the decrease in content of phosphate groups by measuring the nominal capacity of the ion exchanger. After 12 h of operation the capacity had decreased from the original value of 2.28 to 2.12 mequiv./g. The esterification column still worked with a high efficiency after 25 l of the reaction mixture had been passed through it. After the end of the whole experiment (total operation period 275 h), the mean capacity of the bed was 1.7 mequiv./g, but this value was not evenly distributed over the column. The upper part was most (1.58 mequiv./g), the middle part less (1.82 mequiv./g) and the lower discharge part least exhausted (1.94 mequiv./g).

A blank experiment, to determine the contribution of autocatalysis to the esterification, was carried out in the same column but packed with inert glass balls of diameter 0.8 mm. The highest degree of autocatalysis was found at 80°C, flow through 19 ml/h and acid:alcohol ratio of 1:1:2; the degree of conversion of acid was

11.8%. The kinetics of the esterification reaction catalyzed by the ion exchanger were also followed and compared with those in the presence of the equivalent amount of free phosphoric acid. Both reactions were found to be of the second order (with respect to the concentrations of acetic acid and *n*-butane), that catalyzed by the ion exchanger proceeding more than two times faster (rate constant $k_2 = 5.49 \times 10^{-4}$ l/mol · min) than the reaction catalyzed by the acid ($k_2 = 2.06 \times 10^{-4}$ l/mol · min). A more detailed analysis of these results can be found in ref. 18.

The cation exchanger of nominal capacity 2.20 mequiv./g was used for a continuous inversion of saccharose, *i.e.*, for the hydrolysis of this disaccharide to glucose and fructose. A 24% solution of saccharose in distilled water was pumped through the column at rates ranging from 20 to 200 ml/h and temperature was maintained at 50, 60, 70°C or higher. The degree of conversion of saccharose was 100% at flow-rates up to 70 ml/h at 70°C and the resulting solution remained colourless. The conversion was almost complete also at a flow-rate of 100 ml/h and 70°C. At higher temperature, *e.g.*, 90°C, the processed solution was coloured brown. A part of the functional groups of the catalyst was also lost during the preparation of invert sugar, the content of phosphorus in the products being in the range $2 \times 10^{-3} - 5 \times 10^{-3}$%. Upon conclusion of the experiments, *i.e.*, after 5 days, when 13–14 l of the reaction mixture had continuously passed through the column, the nominal capacity decreased in the upper part of catalyst from the initial 2.20 to 0.85 mequiv./g at a depth of 4 cm from the surface to 1.10 mequiv./g and at a depth of 20 cm only to 1.84 mequiv./g. The lower layers of the catalyst bed were practically unaffected.

The volume velocity of catalysis, *i.e.*, the ratio of the flow-rate (per hour) to the volume of catalyst (in the same units), was 0.8–1.0. This value is interesting from the point of view of possible practical applications. However, the chemical instability at the higher temperatures required for continuous catalysis represents a problem.

## CONCLUSIONS

The phosphate derivatives of Spheron may best be prepared by phosphorylation of Spheron with phosphoryl chloride in the presence of a soluble base. These medium-acidic ion exchangers absorb proteins under static conditions, even at lower nominal capacities, and can be prepared with relatively high nominal capacities of 3.5–4 mequiv./g. They proved suitable for medium-pressure chromatography of proteins at ambient or lower temperature. Under such conditions, they may be repeatedly regenerated and reused. Enzymes chromatographed on these ion exchangers retain full activity. Spheron phosphates were also used as immobilized acids in the catalysis of alcohol esterification with organic acid and for the inversion of saccharose to glucose and fructose. At higher reaction temperatures, small amounts of phosphorus are lost, particularly in the upper parts of the column.

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