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The Separation of Buffer Salts at Gel Filtration

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During gel filtration of proteins, artefacts were observed which led to the observation that the salts of the buffers used were separated simultaneously with the proteins. Tests with pure salt solutions indicated that during sieving as well as during displacement chromatography all kinds of salt fractionation could occur. Even pure water "sieved" through the column produced several conductivity peaks when the column was in equilibrium with a buffer containing polyvalent ions.

Actual separation of both simple anions and cations in mixtures have been analytically demonstrated under conditions varying from 0.1 N acetic acid to 0.1 N ammonium hydroxide in the column. It is suggested that ions retarded by the gel matrix give the gel the property of an amphoteric ion exchanger with respect to the non-retarded ions outside the matrix.

In an experiment to fractionate and desalt [1] solutions containing enzymatically digested casein [2] (cheese preparation) through columns of Sephadex G 25*, we found that the inorganic salt ions present were fractionated simultaneously. This caused artefacts in the form of false peptide peaks. The reason for this is probably that the phosphopolypeptides formed micells at certain points in the column where a combination of pH-decrease and Ca-increase occurred and thus moved at a greater speed than otherwise.

A series of experiments was carried out in order to obtain some understanding of this undesirable distribution of salts during fractionation. The immediate purpose was to eliminate the disturbances, although the results may quite possibly be applicable to the intentional fractionation of similar substances.

A chromatograph column, 2 × 110 cm, was packed with Sephadex G 25 treated according to the manufacturer's instructions [3]. To permit the continuous registration of changes in conductivity of the eluate, the eluate was led through the conductivity cell of a Conductolyzer ** at 25°C ± 0.01°C and the changes were measured and registered. In some instances, the eluate was led further to a fraction collector.

* Manufactured by AB Pharmacia, Uppsala, Sweden.
** Manufactured by LKB-Produkter AB, Stockholm 12, Sweden.

When boric acid was not included in the buffer, the Sephadex column was kept at somewhat varying room temperatures. When boric acid was present, it was necessary to maintain a constant temperature since the equilibrium of the Sephadex-boric acid complex is markedly dependent on temperature, and the only possibility of obtaining straight base lines is therefore to keep the temperature constant. The rate of flow through the column was kept constant in all experiments at 30 ml/h. The input of the Conductolyzer was connected in parallel with a resistor of 8.2 kΩ, so that the extreme changes in conductivity which occurred in certain cases could be kept within the limits of the diagram paper. The position of the base line and the sensitivity of the recording instrument were adjusted according to experience to give suitable curves. The paper speed of the recorder was kept at 1 cm/h in all experiments except for those illustrated in Fig. 4.

The experiments were divided into four parts: displacement of one buffer in the column by another; filtration of distilled water through columns in equilibrium with buffers of various ionic types; filtration of 0.1 N NaCl under the same conditions as above; and filtration of mixtures of anions or cations through a column equilibrated with 0.1 N acetic acid or 0.1 N ammonia.

The buffer solutions used were: 0.1 N NaOH + citric acid to pH 6.7; phosphate buffer, μ 0.1, at pH 7.5; 0.1 N HN_4OH; 0.1 N acetic acid; 0.1 N trishydroxymethyl-aminomethane ("Tris"); 0.1 N NaOH + boric acid to pH 10.5; Aronsson-Grönwall's high resolution buffer [4], 0.1 N with respect to "Tris" (12.10 g/l "Tris", 1.20 g/l EDTA, 0.92 g/l boric acid), and a couple of equiconductive buffers of the same pH-value, obtained through neutralizing 0.1 N NaOH with conc. H_3PO_4 or citric acid to pH 7.00.

The procedure in the experiments was to adjust the column to complete equilibrium with the buffer used by letting the buffer run through until the conductivity of the eluate remained constant for at least 2 h. By means of the customary chromatographic technique, 10 ml of the test solution was then applied to the column. Elution with the buffer followed immediately and the recorder was started simultaneously. The procedure in the displacement tests was analogous. The ions in the fractions were identified with the aid of the usual analytical techniques.

Some of the conductivity curves obtained have been re-traced and are given in Figs. 1—4. This was done in order to correct for some maladjustments during some of the first experiments before sufficient experience had been acquired.

Fig. 1 gives the results when water was filtered through the column. When the water passed the conductivity cell, the recorder indicated lower conductivity. Depending on the degree of diffusion of water and salts, the peak registered will have the basic character of an ordinary Gaussian curve rather than being rectangular. However, the water curves shown in the figure have different shapes and deviate from a Gaussian curve in some instances, indicating that the water does not pass through the column without interference with the gel and the buffer salts. This is demonstrated more clearly by the fact that, for each of the different buffers used, there are marked variations in the flow rate of the water through the column in relation to the rate for the eluating buffer. Migration is fastest in the citrate buffer and slowest in the borate buffer. In a composite buffer such as the Aronsson-Grönwall buffer, the water

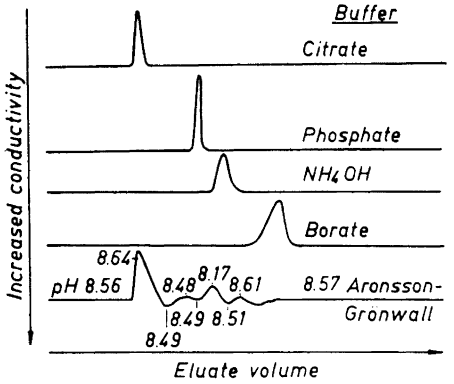

Fig. 1. Gel filtration of 10 ml water through a column packed with Sephadex G 25 in equilibrium with the buffers stated. The interaction between the water and the gel is illustrated by the different shapes and positions of the water peaks in the conductivity curve. Separation of the components in the Aronsson-Grönwall buffer is indicated by the shape of the curve and the pH-value of the eluate corresponding to the peaks obtained.

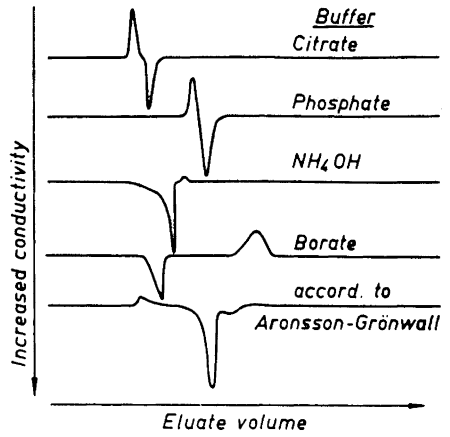

Fig. 2. Gel filtration of 10 ml 0.1 N NaCl under the same conditions as in Fig. 1. When buffers with polyvalent anions are used, the shape of the curve indicates the separation of water and NaCl. Peak heights correspond to a conductivity change of 2—10 %.

will even give rise to a whole series of peaks which represent conductivities both higher and lower than that of the eluating buffer. The eluate fractions corresponding to these peaks were found to vary in their pH-values and buffer capacity, which confirmed fractionation of the buffer when the water passed through the column. The pH-values obtained have been included in the figure.

Fig. 2 illustrates the the result of the corresponding gel filtration of 0.1 N NaCl. Monovalent buffers gave distorted Gaussian curves of the type exemplified by the ammonium hydroxide curve. Polyvalent anion types, on the other hand, gave complex curves. Citrate, phosphate, and borate buffers all gave two peaks for the gel filtration of NaCl, the one showing a quite low conductivity and the other showing a high conductivity in relation to the conductivity of the buffer before and after the peaks. In the case of the citrate and phosphate buffers, the high conductivity peaks follow immediately after the low conductivity peaks. In the case of the borate buffer, the high conductivity peak appears first and is followed by a long interval before the low conductivity peak is apparent. When the Aronsson-Grönwall buffer is used, the curve is again rather complex. However, it is not possible to discard *a priori* the assumption that the single peaks observed, such as with NH_4OH, consist of superimposed curves of different types. It would therefore be too presumptuous to draw extensive conclusions from these observations although it is

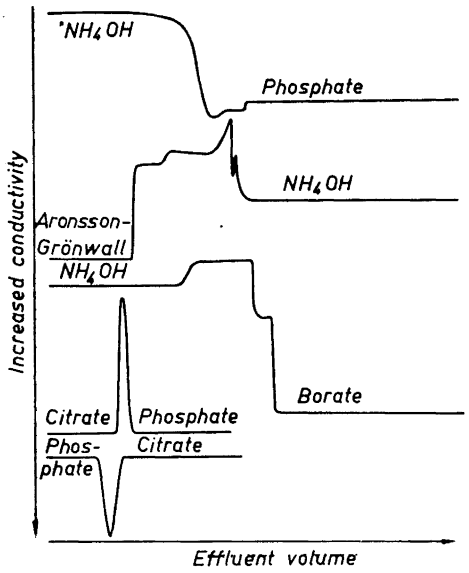

Fig. 3. Displacement of one buffer in the Sephadex G 25 column by another. The steps in the conductivity curves indicate a strong interaction between the buffer ions and the gel or the original ions retarded by the gel.

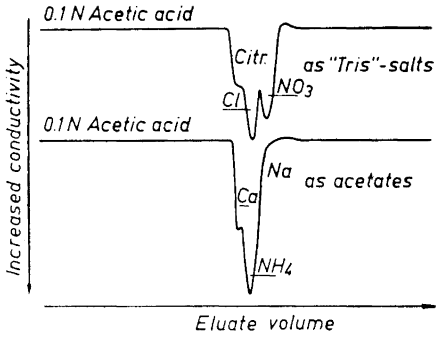

Fig. 4. Under similar conditions, Sephadex G 25 is able to bring about at least a partial separation of both anions and cations in the mixtures. The gel appears to act as an amphoteric ion exchanger.

clear that water and NaCl do become separated during the passage through the column.

In the displacement of one buffer in the column by another, the curves obtained show more or less distinct steps. This is illustrated by some examples in Fig. 3. Even here, each of the superimposed effects appears as a step which is modified in the direction of either low or high conductivity.

In one experiment, buffers having almost equivalent conductivity, ionic strength and pH were permitted to replace each other. The buffers used were sodium phosphate and sodium citrate, both being made by neutralizing 0.1 N NaOH with the respective acids in concentrated or solid form to pH 7.00 $\pm$ 0.005. When the Sephadex column is saturated with the citrate buffer and the displacement made with phosphate buffer, a solitary sharp peak with low conductivity appears (water?). When the displacement procedure is reversed, there is a similar single peak but this is in the direction of increased conductivity, indicating that the salts have partially migrated over each other.

Finally, in order to demonstrate the separation of ions and groups of ions in the eluates, several experiments were performed, some of which are presented in Fig. 4. Two experiments were carried out with the column saturated with 0.1 N acetic acid. In one of these, a mixture of citric acid, hydrochloric acid and nitric acid was filtered through the gel, all of these substances at 0.1 N

and dissolved in 0.1 N acetic acid. The fractions obtained were analyzed and showed a relatively good separation of the acids. The separation was improved if the solution was made 0.3 N with regard to "Tris", before filtration. The citric acid is the first to appear, the other acids following as shown in Fig. 4. When not neutralized with "Tris" the citric acid appeared last.

In the second of these experiments, Ca^{2+}, NH_4^+ and Na^+ were separated from each other as acetates. There was poor separation, however, between NH_4^+ and Na^+. In this case, too, factors were observed which reversed the order of the ions (presence of other anions, pyridine, etc.).

DISCUSSION

The phenomena observed are associated with some form of retardation of the ions moving through the column. It is not possible to state definitely whether this retardation is caused by filtration according to molecular size, partition chromatography, ion exchange chromatography or by other effects. When buffers containing boric acid are used, a marked ion exchange effect is to be expected since the boric acid is bound to the Sephadex gel and gives a relatively strongly ionized complex. A possible explanation of the effects occurring with the other types of buffers might be that the anions and cations which are more or less bound by the gel matrix give the gel the character of amphoteric ion exchanger in its relationship to the mobile ions outside the gel. In this case, the amphoteric ion exchanger would exhibit an unique property in that its ionized groups would migrate steadily through the column with a characteristic migration rate of their own, determined by the ion form and size.

Ion exchange properties of the gel *per se*, as discussed by Miranda *et al.*[5], seem less likely. Even if the ion exchanger is amphoteric, one can scarcely expect the simultaneous separation of both anions and cations within the wide pH interval provided by 0.1 N HAc—0.1 N NH_4OH.

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