ARTIFICIAL ZONING IN ANION EXCHANGE CHROMATOGRAPHY

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INTRODUCTION

The risk of chromatographic artifacts being formed in protein chromatography due to the interaction of anion exchangers and alkaline buffers with atmospheric carbon dioxide has been studied previously[1,2]. In one-step development chromatography at a fixed pH with chloride as the developing ion, it was found that an anomalous chloride step appeared together with a carbonate zone and a pH "hump". Experimental data were collected[2], but no definite explanation of the anomalous chloride step was presented. It was, however, predicted that under certain conditions similar phenomena might also appear in gradient development and in chromatography with mixed buffers.

In the present paper the anomalous step has been shown to arise not as a part of the developing front but from the chloride adsorbed on the column during equilibration. The deformation of gradients by passage through the column has been studied; and in a gradient development with mixed buffers highly purified bovine carbonic anhydrase has been split up into two active peaks that seem to be identical.

EXPERIMENTAL

Buffer systems

Tris-(hydroxymethyl)-aminomethane–hydrochloric acid (Tris–HCl) and tris-(hydroxymethyl)-aminomethane–carbon dioxide (Tris–CO_2) buffers were prepared by dissolving the appropriate amount of Tris in common distilled water, and then adding concentrated HCl or bubbling CO_2 through the solution until the desired pH was reached. Buffer concentrations are given as molarities of Tris.

Sodium tetraborate–hydrochloric acid buffer was prepared by dissolving the tetraborate in common distilled water and adjusting the pH with concentrated HCl. Buffer concentrations are given as molarities of tetraborate.

Adsorbent

Diethylaminoethyl(DEAE)-cellulose with a nitrogen content of 14.0 mg/g was prepared according to PETERSON AND SOBER[3]. Before use it was washed thoroughly with 1 *N* HCl, water, and 1 % NaOH. In order to get a suitable flow-rate, the smallest particles were removed by suspending the ion exchanger in water and sucking off the supernatant suspension after the larger particles had settled.

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Chromatography

The ion-exchanger suspension was deaerated in a suction flask and filled into the column under a water pressure of about twice the column length. Before an experiment the column was always washed through with 1 % NaOH, and then with 20–30 times its dead volume of starting buffer, equilibration being checked by measuring the pH of the effluent. When the starting buffer contained chloride ions, the chloride concentration was also determined.

In all the experiments described 19 ml columns of the same dimensions (24 × 1.0 cm) were used. Their dead volume was about 8 ml. The flow rate was kept at 15 ml/h and the fraction volumes were 1.0 ml in one-step development and 2.0 ml in gradient development chromatograms. No special precautions were taken to protect the columns against atmospheric carbon dioxide.

For gradient development a mixing chamber provided with a magnetic stirrer and a tightly-fitting dropping funnel was directly connected to the top of the column. At the start of a chromatogram the mixing chamber was filled with starting buffer and the dropping funnel with developing buffer. As the buffer volume in the mixing chamber was constant during the run, the ionic concentrations in the effluent from the mixing chamber were exponential functions of the effluent volume.

Bovine carbonic anhydrase

This was prepared by Dr. S. LINDSKOG using a procedure which yields a highly purified enzyme, which is homogeneous in free electrophoresis and in the ultracentrifuge[4].

Analytical methods

Chloride analyses were made by the mercurimetric method, the end-point pH in titration being kept at about 2.0 or lower[5]. Concentrations are expressed as millimoles per litre.

Carbonate concentrations were determined in the following way. To 1.0 ml of 0.025 *M* $BaCl_2$ in a small test-tube (8 × 1.1 cm) 0.5 ml of the sample was added. The tube was corked carefully, and after being shaken gently it was left at room temperature (22°) for 2 h. The $BaCO_3$ precipitate was spun down, and 0.5 ml of the supernatant was added to 0.5 ml of 0.02 *M* K_2CrO_4 which had been adjusted to an alkaline pH by the addition of carbonate-free NaOH. Remaining barium ions were thus precipitated as $BaCrO_4$. After centrifugation 0.1 ml of the supernatant was diluted with 1.0 ml of distilled water (if necessary, further dilutions were made), and the absorbancy was measured in a Beckman B spectrophotometer at 372 mμ using a 1-cm semi-micro cell and water as a blank. Together with each set of carbonate determinations a new standard curve (with Na_2CO_3 which had been dried at 300° for 1 h) was always made. Concentrations are expressed as millimoles per litre.

Borate concentrations were estimated by a semi-quantitative adaptation of the qualitative curcuma-paper test[6]. The fractions were diluted to fit into a standard scale of successive dilutions of $Na_2B_4O_7$, which was made simultaneously. Five different dilutions of each fraction were tested, and the values plotted are averages. Concentrations are expressed as millimoles of tetraborate per litre.

RESULTS

Figs. 1 and 2 show one-step development chromatograms on the same column with different starting buffers but with the same eluting buffer. In the first experiment the column had been equilibrated with 0.005 *M* Tris–HCl of pH 9.0; in the second with 0.005 *M* Tris–CO_2 of the same pH. In both cases elution was performed with 0.3 *M* Tris–HCl of pH 9.0. It can be seen that the pH and carbonate concentration curves in Fig. 1 do not differ significantly from those in Fig. 2, whereas the anomalous chloride step in Fig. 1 (tubes 13–21) is completely missing in Fig. 2.

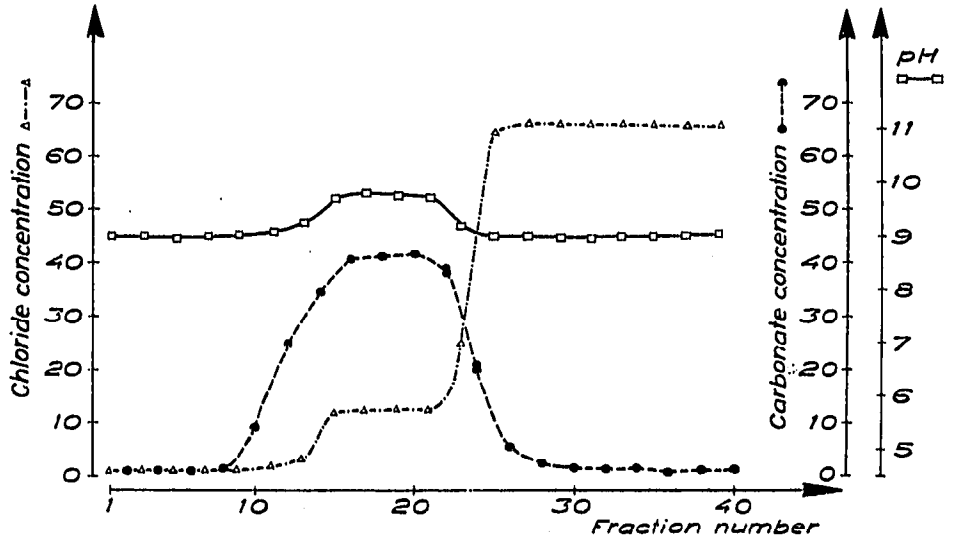

Fig. 1. One-step development chromatogram on a 19 ml column (24 × 1.0 cm) of DEAE-cellulose. Starting buffer 0.005 *M* Tris–HCl of pH 9.0. Developing buffer 0.3 *M* Tris–HCl of the same pH. Fraction volumes about 1.0 ml. Chloride (△–·–△) and carbonate (● – – – ●) concentrations are expressed as mmoles/l.

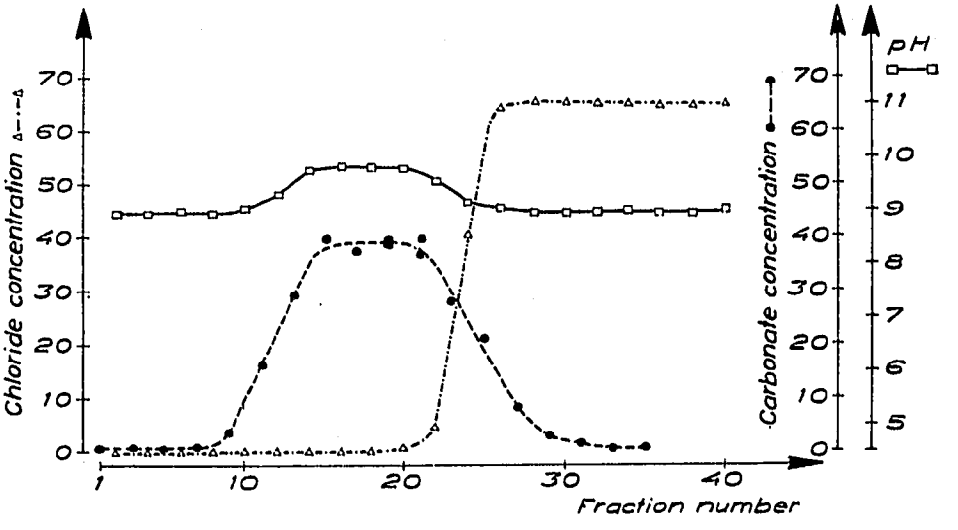

Fig. 2. One-step development chromatogram on the same column as in Fig. 1. Starting buffer 0.005 *M* Tris–CO_2 of pH 9.0. Developing buffer 0.3 *M* Tris–HCl of the same pH. Fraction volumes about 1.0 ml. Chloride (△–·–△) and carbonate (●–––●) concentrations are expressed as mmoles/l.

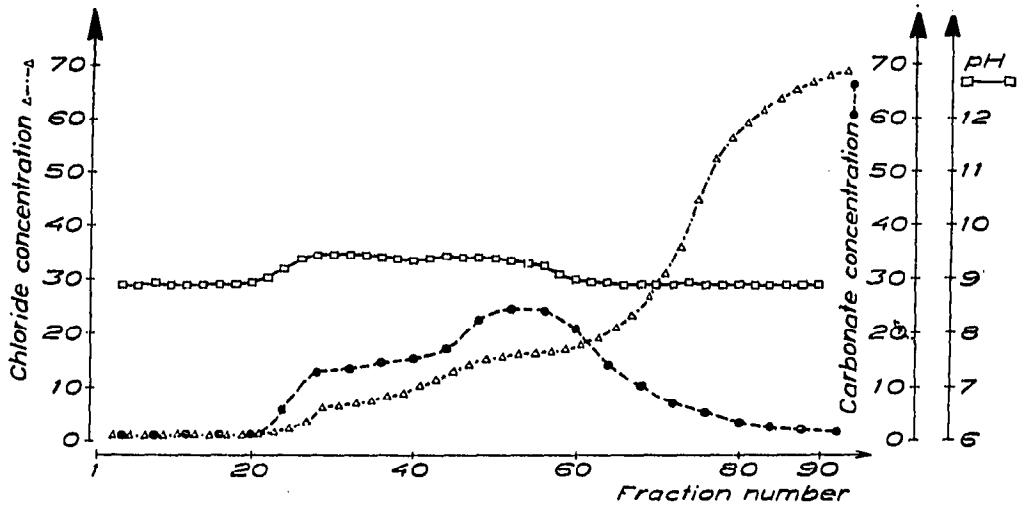

Fig. 3. Chloride-gradient development chromatogram on a 19 ml column (24 × 1.0 cm) of DEAE-cellulose. Starting buffer 0.005 *M* Tris–HCl of pH 8.9. For the gradient device, see the text. Fraction volumes about 2.0 ml. Chloride (△–·–△) and carbonate (●———●) concentrations are expressed as mmoles/l.

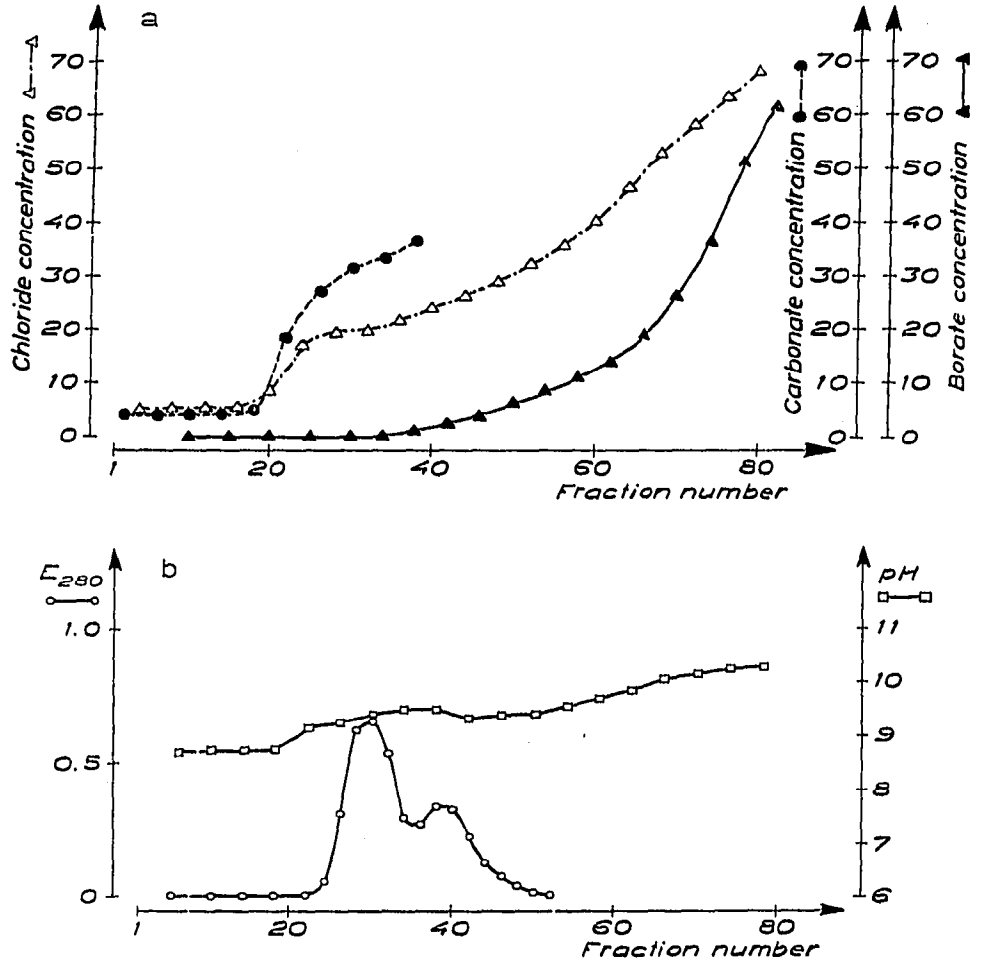

Fig. 4. Gradient development chromatography of 9.2 mg of bovine carbonic anhydrase with mixed buffers. The same column as in Fig. 3. Starting buffer 0.005 *M* NaCl in 0.02 *M* Tris–CO_2 of pH 8.7. For the gradient device, see the text. Fraction volumes about 2.0 ml. Chloride (△–·–△), carbonate (●———●) and tetraborate (▲—▲) concentrations are expressed as mmoles/l.

A chloride-gradient development chromatogram is shown in Fig. 3. The column had been equilibrated with 0.005 *M* Tris–HCl of pH 8.9, and at the start of the chromatogram the mixing chamber contained 145 ml of this buffer. The developing buffer was 0.5 *M* Tris–HCl of the same pH. Under these conditions the chloride-concentration gradient flowing into the column was only slightly convex upwards. The figure shows that passage through the column changed the gradient considerably.

The chromatogram shown in Fig. 4 was obtained on the same column as that shown in Fig. 3. Equilibration had been carried out with 0.005 *M* NaCl in 0.02 *M* Tris–CO_2 of pH 8.7. A solution of 9.2 mg of bovine carbonic anhydrase in 1 ml of starting buffer was applied to the column. When the enzyme solution had run into the bed, the eluting gradient was started. The mixing chamber contained 145 ml of starting buffer. The developing buffer was 0.5 *M* with reference to Tris–HCl of pH 8.7 and 0.1 *M* with reference to $Na_2B_4O_7$ and had been adjusted to pH 10.4 by the addition of 1 *N* NaOH. In Fig. 4b it can be seen that the carbonic anhydrase has been split up into two peaks. These were found to have the same specific activity and the same mobility in paper electrophoresis in Tris–HCl buffer at pH 8.7. Rechromatography of each peak under the same conditions as described above showed that both of them were split up again in a similar way.

DISCUSSION

Analytical methods

In a previous paper[5] it was shown that mercurimetric chloride determinations carried out in the usual way are seriously disturbed by the presence of high concentrations of Tris buffer. To avoid this the end-point pH should be kept at about 2.0 or lower. For some fractions from the chromatogram shown in Fig. 4 the chloride values obtained by this procedure were compared with those obtained after ashing the samples together with an excess of sodium hydroxide. The difference found was in no case higher than 3 %.

The standard curves for carbonate determinations showed a deviation from stoichiometric linearity. At low concentrations the barium carbonate precipitation seemed to be incomplete; at high concentrations the absorbancy observed was higher than expected, which might be interpreted as an incomplete precipitation of the low concentrations of barium chromate.

In order to avoid interference by atmospheric carbon dioxide the chromatographic fractions were tested for carbonate as soon as possible, and the reagent solutions used were not older than one day. The method is not very accurate for concentrations lower than 0.005 *M* or higher than 0.04 *M*, but it is much more reliable than the common turbidity tests, and for testing a large number of fractions at the same time it is extremely convenient. A disadvantage is that carbonate cannot be determined in the presence of tetraborate, as this ion will also be precipitated by barium chloride. An attempt to obtain reliable standard curves for carbonate in the presence of known amounts of tetraborate was not successful.

One-step development at a fixed pH

A comparison of the chromatographic patterns in Figs. 1 and 2 shows that the anomalous chloride step in Fig. 1 must have arisen from the chloride adsorbed on the

column during equilibration. When the displaced carbonate zone travels down the column, part of the equilibration chloride is forced out from the adsorbent. As the affinity of chloride is higher than that of carbonate, the chloride will not, however, appear as a displaced zone in front of the carbonate peak but will instead tend to lag behind. In the frontal part of the carbonate peak more chloride will be desorbed, and in this way the stationary anomalous chloride step is developed.

It is obvious that the carbonate zone will have a similar influence upon adsorbed protein. If the protein affinity is low, the carbonate zone will act as a carrier and displace the protein. But if the affinity of the protein is high enough, only part of it will be displaced by the carbonate, while the rest will be eluted by the main chloride step. The result would thus be the same as with a two-step development where the concentration of the first step is just within the elution range of the protein. The actual situation is, however, somewhat more complicated, as the pH is not constant but changes directly with the carbonate concentration. These variations in pH certainly affect the ion exchange capacity, and they may also change the protein affinity.

We have also studied chloride adsorption isotherms at different pH values to see whether the chloride affinity factor is dependent on pH. At low concentrations it was practically impossible to get coinciding adsorption and desorption curves, which means that a true equilibrium could not be attained. Significant differences were nevertheless found between curves obtained at different pH values. Fig. 2 shows that this cannot be of any importance for the appearance of the anomalous step, but it is possible that the slope of the main chloride front is influenced by a variation of the affinity factor with pH.

Chloride-gradient development at a fixed pH

The deformation of a concentration gradient during passage through the column is dependent upon the curvature of the adsorption isotherm. In the chromatogram shown in Fig. 3 the influent gradient was slightly convex upward; the shape of the effluent gradient indicates that the chloride adsorption isotherm should be strongly curved, which in fact it is. For the tubes 29–65 the chloride concentrations are higher than what would be expected from the isotherm studies, but this can be explained, as before, by the presence of the carbonate zone. The influent chloride gradient breaks through in tube 79, and from that point on the influent and effluent curves coincide.

Gradient development with mixed buffers

The chromatogram shown in Fig. 4 is a good illustration of the difficulties encountered when using a complex buffer system. The anions in the starting buffer were chloride, carbonate and hydroxyl ions, while the developing buffer contained chloride, tetraborate and hydroxyl ions (and the carbonate absorbed from the air). In the influent solution the carbonate concentration was decreasing with time; the concentration of all the other anionic species was increasing. As tetraborate interferes with the carbonate determinations, these were not carried out after tube 38.

Bovine carbonic anhydrase was chosen as a test substance owing to its purity and its favourable adsorption properties. The results of activity tests, paper electrophoresis, and rechromatography of the two enzyme peaks indicate that the heterogeneity observed (Fig. 4b) is not a real one. In a case like this it would be unjustified to conclude from the absorbancy pattern alone that two active forms of the enzyme

have been found. The irregularity of the effluent pH gradient should be taken as a warning against rash interpretations, and, if possible, the effluent anion concentrations should be measured. In Fig. 4a it can be seen that a high carbonate peak with a concomitant chloride step is eluted in the same region as the first enzyme peak (tubes 22–34); when the tetraborate appears in the effluent and the chloride concentration begins to rise again, the second enzyme peak is eluted. It is thus very likely that the two enzyme peaks are identical and that the splitting is a consequence of the mechanism described above. However this may be, we should like to point out that in situations of this kind such a possibility should be borne in mind. Rechromatography of each peak under the same conditions should be made, and the properties of the two peaks should be compared in as many different ways as possible. If there is no other way of distinguishing them, the evidence for heterogeneity must be considered very weak.

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SUMMARY

1. A study has been made of step-wise and gradient development in anion exchange chromatography.

2. In one-step development the anomalous step previously studied has been shown to arise from the anions adsorbed on the column during equilibration.

3. The deformation of concentration gradients by passage through the column has been demonstrated.

4. By the use of a combined pH and concentration gradient with mixed buffers highly purified bovine carbonic anhydrase has been split up into two active peaks, which are probably identical.

5. The possibilities of obtaining artificial protein peaks in one-step and gradient development chromatography on anion exchangers are discussed.

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