ION EXCHANGE CHROMATOGRAPHY OF PROTEINS

ARTIFACT SEPARATIONS RESULTING FROM INADEQUATE pH CONTROL*

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The technique for separation of proteins by ion exchange on substituted cellulose has been used extensively since it was first introduced by PETERSON AND SOBER[1,2] in 1956. Since then the technique has been the subject of at least two general reviews[3,4]. For the most part these reviewers deal with the mechanics of the method. However, PORTER[4] mentions that basic knowledge about the ion exchange process is rather meagre and that the anomalies and difficulties which may be expected are relatively unknown. In the literature there have been several reports of anomalous results, some of which are probably related to the ion exchange process itself and some are not.

NIMMO *et al.*[5] and VEDER AND PASCHA[6] have observed significant pH changes in the effluent, during chromatography of proteins on cellulose ion exchangers, which were likely a result of ion exchange processes. The effect of the pH changes is, however, not clear because both of these reports deal with only one set of chromatographic conditions and no attempt was actually made to show the effect of the variable pH on the resulting chromatograms. Furthermore the pH change observed by VEDER AND PASCHA[6] occurred when a stepwise change was made in the eluting solvent. This may be related to the "double-fronting" phenomenon described by BJÖRK[7] in 1959. Since the possibility of obtaining double peaks of the same material by stepwise elution chromatography was first pointed out in 1952 by ALM, WILLIAMS AND TISELIUS[8], and has been re-emphasized several times[3,4,9] since then, this method will not be considered further. Instead it will be the object of this paper to deal with the possibility of artifact separations resulting from inadequate pH control during gradient elution of proteins from ion exchange celluloses. This investigation was undertaken when some preliminary experiments indicated that each successive peak of wheat protein eluted from carboxymethyl (CM) cellulose corresponded to a progressive lowering of the pH of the effluent solution.

METHODS AND MATERIALS

The chemicals used in this study were generally of the best available reagent grade. The wheat flour was milled in this laboratory under carefully controlled conditions from a sample of a pure variety of hard red spring wheat. The flour and all the solutions used for this work were stored in a cold room (2–3°).

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Extraction of flour proteins

The extraction of proteins from the flour was conducted in the cold room. Flour (75 g) was slurried in a Waring Blendor under nitrogen with 210 ml of a 0.0100 *M* sodium pyrophosphate solution which also contained 0.00250 *M* disodium ethylenediaminetetraacetate (EDTA) and was adjusted to pH 7.0 with hydrochloric acid. Toluene (0.1 ml/l) was added as a preservative. A similar solution without the EDTA has been used by COATES AND SIMMONDS[10]. The slurry was centrifuged in the cold at approximately 8000 g for 15 min; the supernatant was collected and the residue slurried again with the pyrophosphate buffer. In all the flour was subjected to four successive extractions with 210, 135, 130 and 125 ml respectively of pyrophosphate buffer. The protein content of the combined extracts was determined by the Kjeldahl method.

Column chromatography

Solutions. For experiments on CM cellulose, solutions were prepared from disodium malonate and other salts and were adjusted to the desired pH with dilute malonic acid solution in order to maintain a known sodium ion concentration. All solutions for chromatography contained $1.00 \cdot 10^{-4}$ *M* EDTA and 0.1 ml/l of toluene in addition to the other indicated constituents.

Cellulose. Celluloses were commercial preparations. Newc ellulose was washed alternately with 0.1 *N* HCl and 0.1 *N* NaOH at least five times or until no more colored material was removed from the cellulose. Fine material was removed by decantation after the major portion of the cellulose had settled from a dilute suspension.

Since the cellulose columns were used for many experiments, it might be useful to describe the regeneration procedure. After each ion exchange experiment, the column (2×38 cm) was washed with 2 l of 0.1 *N* NaOH to remove any residual protein, followed by 0.5 l of 0.0500 *M* disodium malonate pH 5.5 to equilibrate the cellulose to pH 5.5. The cellulose was then withdrawn from the column, diluted with distilled water to 1 l, and allowed to settle. Fines were removed by decantation, the suspension was diluted to 0.7 l with water and dissolved gases were removed by stirring under vacuum for a few minutes. The degassed, dilute suspension was poured into the column with the aid of a 40 cm extension tube. The settled cellulose was washed first with 0.25 l of 0.0500 *M* disodium malonate pH 5.5 and finally with 1.7 l of 0.00500 *M* disodium malonate pH 5.5, the starting buffer in experiments 1 and 2, to complete the equilibration of the cellulose.

In experiment 3, after 2.5 l of starting buffer was pumped through the column, the effluent pH was still approximately 6. This was corrected by pumping 0.5 l of 0.00500 *M* disodium malonate pH 5.5 through the column, followed by 0.3 l of 0.00100 *M* disodium malonate, 0.00800 *M* NaCl starting buffer. The final effluent pH was 5.6.

Protein separation. A schematic diagram of the apparatus is shown in Fig. 1. The gradient device consists of four identical cylindrical plastic vessels in hydrostatic equilibrium interconnected by small bore rubber tubing, and is identical in principle to devices described by PETERSON AND SOBER[11]. The tubing which carries the buffer from the gradient vessels to the pump and from the pump to the rest of the apparatus is 1/16 in. inside diameter Tygon tubing. The glass fibre plugs in the Tygon line

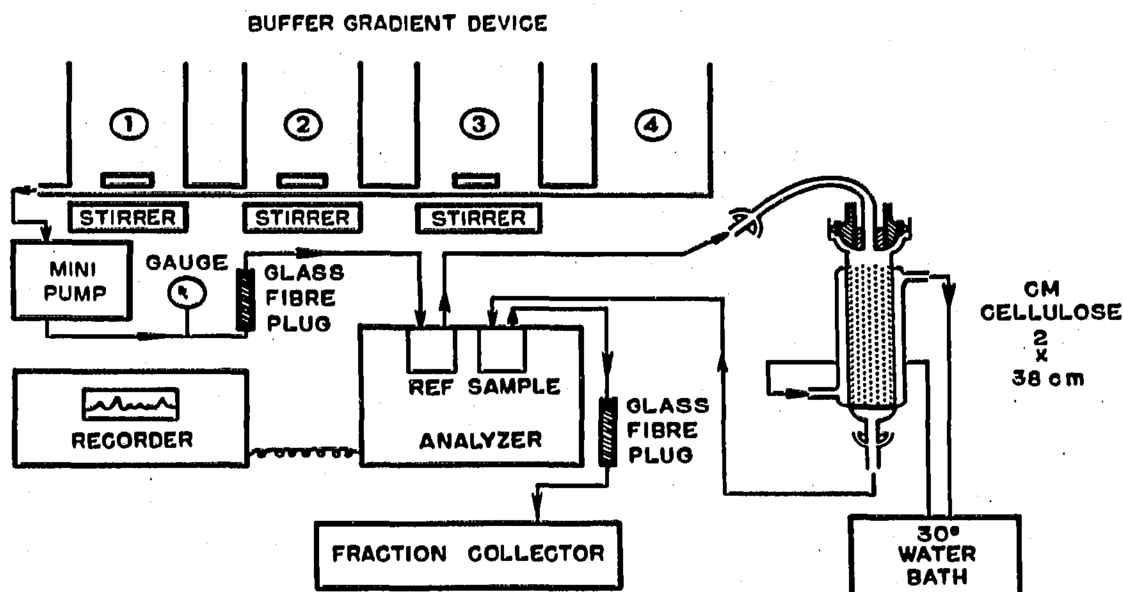

Fig. 1. Schematic diagram of ion exchange chromatography apparatus.

provide a slight back-pressure which is necessary to maintain a constant flow rate from the pump and also to prevent the formation of air bubbles in the sample cuvette. The buffer pump was of the positive displacement piston type; the flow rate in all experiments was 1.25 ± 0.005 ml per min.

The Vanguard Ultraviolet Analyzer was equipped with 0.66 cm quartz cells and the diffraction grating was set at 280 mμ to estimate protein in the column effluent. The recorder chart speed was 0.5 in./h. The fraction collector was actuated at 20 min intervals; and thus collected 25 ml samples.

A portion of the flour extract equivalent to 450 mg of protein (Kjeldahl $N \times 5.7$) was dialyzed thoroughly against three changes of the starting buffer. Approximately 30 % of the nitrogen is lost during this step. The dialyzed protein solution was centrifuged to remove a small quantity of insoluble material and then allowed to run into the column under gravity. When all the protein solution had run into the column, the top of the column was rinsed with starting buffer. The headspace was filled with starting buffer, and the same buffer was pumped through the column until all the unadsorbed protein was removed from the column. The pump intake was then switched to the gradient device and the desired gradient of increasing sodium ion was pumped through the column to elute the adsorbed proteins.

The contents of each vessel in the gradient device are given for each experiment in Table I.

The buffer solutions were degassed by stirring under vacuum for a few minutes before the solutions were poured into the gradient vessels. This eliminates the formation of air bubbles in the column. Each vessel contained 500 g of buffer initially. The use of a constant flow rate together with the same sodium ion concentration in a given vessel provides a constant sodium ion gradient in all experiments. Normally at the end of the gradient elution 0.1 N sodium hydroxide was pumped through the column; however, in experiment 3, 500 g of 0.182 M disodium malonate, the limit solution of experiments 1 and 2, was pumped through the column at the end of the gradient before the sodium hydroxide.

TABLE I

MOLAR COMPOSITION OF GRADIENTS USED FOR THE ELUTION OF WHEAT PROTEINS FROM CARBOXYMETHYL CELLULOSE

Experiment		*Vessel*			
		1	*2*	*3*	*4*
1	Disodium malonate	0.00500	0.0405	0.00500	0.182
	Sodium chloride	—	—	—	—
2	Disodium malonate	0.00500	0.00500	0.00500	0.00500
	Sodium chloride	—	0.0710	—	0.354
3	Disodium malonate	0.00100	0.00100	0.00100	0.00100
	Sodium chloride	0.00800	0.0790	0.00800	0.362

Acid phosphatase. Acid phosphatase activity, used to identify a specific protein component, was estimated by a modification of the methods of HOFSTEE[12], and BRANDENBERGER AND HANSON[13]. The substrate *o*-carboxyphenyl phosphate, a commercial preparation, was dissolved in 1.0 *M* sodium acetate (pH 5.5, acetic acid) at a concentration of 1 mg/ml. For the enzyme assay, 1.0 ml of substrate, 1.5 ml water and 0.5 ml of the column effluent were mixed in a quartz spectrophotometer cuvette. The optical density at 300 mμ was measured every minute for 10 min *versus* a blank which contained only substrate, buffer and water. The spectrophotometer sample space was equipped with plates maintained at 25° with circulating water. The relative enzyme activities were determined from the slope of the optical density *versus* time graph.

RESULTS AND DISCUSSION

The results for three experiments are shown in Fig. 2. The sodium ion gradient, which is on the same time–volume scale as the chromatography experiments, was calculated from data given by PETERSON AND SOBER[11]. Acid phosphatase activities are shown on a relative basis; each point represents the change in optical density per minute according to the conditions described in the experimental section. The pH was determined on individual tubes containing 25 ml of the effluent.

The results show quite clearly that as the buffering capacity of the eluant solution is decreased, a higher ionic strength is required to elute a given protein. In experiment 1, with the highest buffer concentration, most of the acid phosphatase was eluted with approximately 0.04 *M* sodium ion. With the progressively weaker buffers in experiments 2 and 3, the sodium ion molarity required to elute the acid phosphatase increased to 0.07 and 0.25 molar respectively.

Furthermore, in experiment 1, the pH decreased to about 5.3 just before the first major protein component was eluted. On the other hand, in experiments 2 and 3, the pH of the effluent was rather variable and the minimums were much lower. In all the experiments, the minimum pH in the effluent was always associated with the elution of a major protein component.

The two phenomena of increasing ionic strength required to elute a given protein and progressive lowering of the effluent pH appear to be related to the

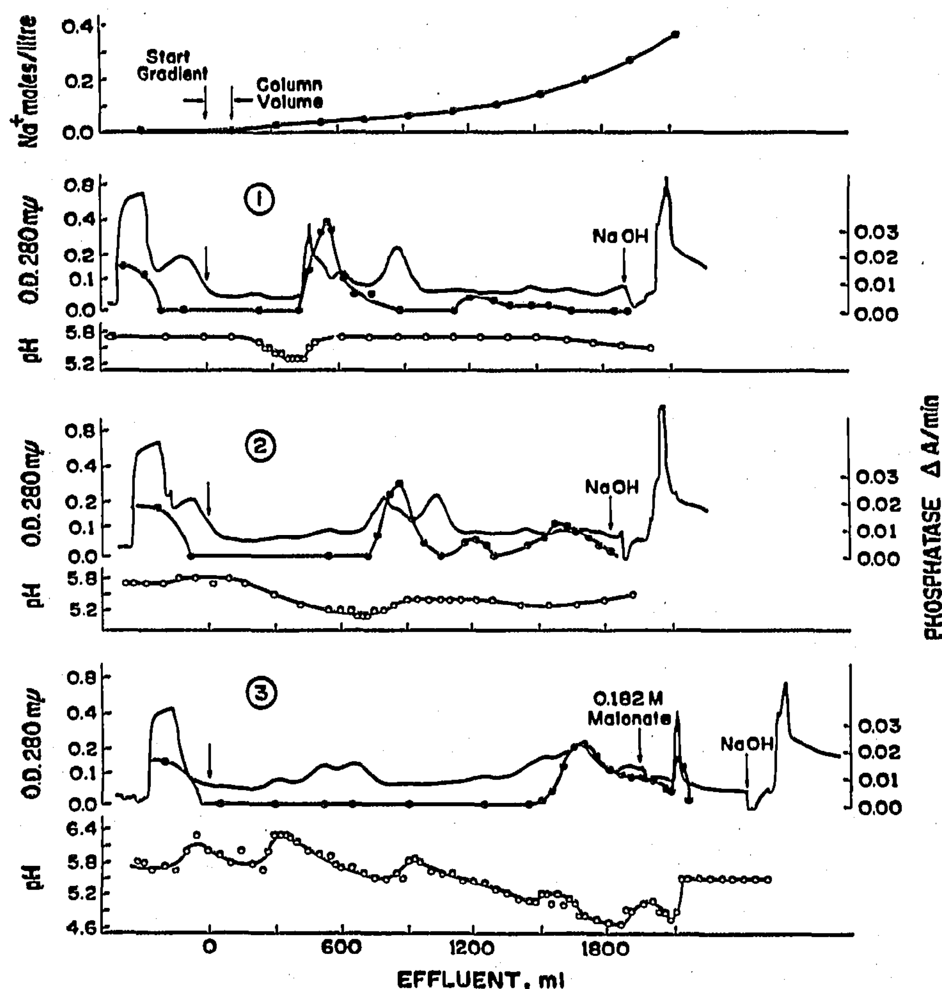

Fig. 2. Results of ion exchange chromatography experiments. Closed circles: sodium ion gradient used in all experiments. Continuous line, no circles: O.D. at 280 mμ, 0.66 cm trace from the automatic recording apparatus. Half-closed circles: relative acid phosphatase activity of individual 25 ml fractions of effluent expressed as ΔA/min at 300 mμ; see text for details. Open circles: pH of individual 25 ml fractions.

relative effectiveness of the buffer in the eluants. This effect may be accounted for in the following way. Proteins are adsorbed onto ion exchange substrates mainly by electrostatic attraction between oppositely charged ionic groups. The adsorption process on CM cellulose involves the displacement of sodium ions from the cellulose by positively charged basic groups on the protein. This process may cause a slight rise in the pH since sodium ions are more basic than any basic protein groups. When proteins are displaced from CM cellulose, the reverse occurs. Thus strongly basic sodium ions in solution displace relatively weakly basic positively charged protein groups from the cellulose. The protein basic groups, when adsorbed onto the cellulose, are probably not in equilibrium with the corresponding free base since the charges are balanced by nearby opposite charges on the cellulose. However, when the protein is displaced by sodium ion, some of the protein basic groups would tend to hydrolyse and thus establish the equilibrium with the free base form. This process will lead to a drop in the observed pH of the solution. In the present work, at a nominal pH of 5.5, one would expect the histidine residues on the protein to be responsible for most of

this effect since the pK for histidine imidazole is about 6 (GREENSTEIN AND WINITZ[14]).

There have been some similar reports in the literature. For example, PETERSON AND CHIAZZE[9] have noted during the separation of human serum proteins on diethylaminoethyl (DEAE) cellulose that a buffer gradient of tris-(hydroxymethyl)-aminomethane (Tris)–phosphate pH 8.6 with a limit concentration of 0.2 M phosphate was actually more effective as an eluant than a gradient composed of ammonium bicarbonate pH 8.2 with a limit of 0.5 M bicarbonate. Since increasing salt concentration and decreasing pH would be expected to reduce the affinity of negatively charged protein carboxyl groups for the positively charged amino groups on the cellulose, the observations are apparently contradictory. The authors suggest that the observations may be accounted for by postulating that a doubly charged phosphate ion is more effective as an eluant than a singly charged bicarbonate ion. At present this postulate is difficult to evaluate. However, tables prepared by DATTA AND GRZYBOWSKI[15] show that the approximate pK's of the various buffer species at 0° are as follows: ammonia 10.1, carbonic acid K_1 6.6, K_2 10.6, phosphoric acid K_2 7.3; the pK of Tris is also given as 8.7 at 5°. PETERSON AND CHIAZZE[9] maintained their ion exchange columns at 5° and thus the pK values at 0° are not entirely appropriate. It would appear, however, from the above data that the Tris–phosphate buffer, which contained Tris and sodium dihydrogen phosphate in an 8:1 molar ratio, was actually many times more effective as a buffer than the ammonium bicarbonate at pH values between 8 and 9. It thus seems likely that the increased effectiveness of Tris–phosphate as an eluant relative to ammonium bicarbonate is mainly a result of the greater buffering capacity of the former salt. However, since these are different salts with different eluant ions some of the specific effects mentioned by PETERSON AND CHIAZZE[9] may also be important.

In the present work only one eluting species was present, namely, sodium ion. Therefore it seems likely that the shift of protein elution to higher salt concentration as the buffer effectiveness decreases is due entirely to the changing buffering capacity of the system. This conclusion is supported by the observation that in all the experiments reported here the pH tends to change in a direction which promotes the retention of protein as proteins are eluted from the column. The present work actually only deals with CM cellulose where the pH is observed to fall as proteins are eluted. On DEAE cellulose one would expect the pH to rise and this effect has been observed by NIMMO *et al.*[5]. Under their conditions of gradient elution, when wheat proteins were fractionated on DEAE cellulose the pH of the effluent rose to 8.5–9.5 during the early part of the elution. The starting pH was not given but the buffer was 0.01 M glycine. A similar solution prepared in our laboratory had a pH of 5.4. While no extensive experiments have been undertaken with DEAE cellulose in the present study, it has been observed that a gradient of sodium phosphate at pH 7.0 is a much more effective eluant than a virtually identical anion gradient composed of sodium chloride in dilute sodium phosphate at pH 7.0.

It seems logical to expect that the exchange capacity of the cellulose would be a factor in the pH control during ion exchange chromatography. Thus a high-capacity cellulose would likely require a higher buffer strength since the protein concentration in the adsorbed phase would be proportional to the cellulose capacity. This may explain why there are some reports[16] in the literature of differences in resolving power between various batches of commercial ion exchange cellulose. Also, the observation

of DINTZIS[17] that only low-capacity celluloses were suitable with a particular gradient seems particularly pertinent.

In the present work the exchange capacity of the CM cellulose was 0.7 meq/g by NaOH titration. The actual cellulose content in the poured column was determined to be 0.1 g/ml; the exchange capacity was thus around 0.07 moles/l. It is of course impossible to calculate on this basis the concentration of buffer which is required to maintain a reasonably constant pH, since this depends upon many factors such as the extent of saturation of the exchanger sites with protein, the amino acid composition of the protein and the extent of ionization of the protein groups which are relatively unknown. It is, however, likely that the very ineffective buffers which have occasionally been used[18–21] would give results which are somewhat ambiguous.

The present work was undertaken specifically to demonstrate the effect of buffer in eluant solutions and not particularly to develop a method for the isolation of wheat acid phosphatase. For this reason no further investigation of the acid phosphatase or other enzymes has been undertaken. Since even in experiment 1 there is a small fluctuation in pH, it seems likely that the first peak eluted by the gradient is a mixture of two or more protein components. Also there is some phosphatase activity not adsorbed under the present conditions. Experiments 1 and 2 have very similar unadsorbed protein peaks; however, the unadsorbed peak in experiment 3 is much smaller. This may possibly be due again to the inability of the weakly buffered solutions to elute as much protein as more effectively buffered solutions. The protein eluted by sodium hydroxide was determined by the Kjeldahl method after the contents of the tubes corresponding to the protein peak were thoroughly dialysed and evaporated to dryness. The results are shown in Table II.

TABLE II

PROTEIN ELUTED BY 0.1 *N* SODIUM HYDROXIDE AFTER SALT GRADIENT ELUTION

Experiment	*Protein* ($mg\ N \times 5.7$)
1	32
2	35
3	21

The smaller amount of nitrogen eluted by sodium hydroxide in experiment 3 is consistent with the fact that the column was eluted with 0.182 *M* disodium malonate at the end of the gradient before the sodium hydroxide elution. In the other experiments, the sodium concentration at the end of the gradient did not reach that level; and therefore it is reasonable that more protein was retained on the column to be subsequently eluted by sodium hydroxide.

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SUMMARY

The ion exchange chromatography of wheat proteins on carboxymethyl cellulose is described. As the concentration of buffer in the eluant is reduced, a higher concentration of sodium ion is required to elute a given protein component; and concurrently the pH of the eluant is observed to deviate from the original pH of the eluant. It is suggested that these effects are related and that they arise from the ion exchange process itself as a result of the non equivalence of eluant ions and charged protein groups. The general conclusion that eluant solutions for protein ion exchange chromatography should be effectively buffered to reduce the possibility of artifact separations also appears to apply to chromatography with DEAE cellulose and other adsorbents.

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