Continuous Fractionation of Protein Mixtures by p H Parametric Pumping:

Experiment

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Continuous pH parametric pumping separations of a haemoglobin-albumin-CM Sepharose system were experimentally investigated. The parapump has a feed containing the protein mixture to be separated, introduced alternately to the top and bottom of the chromatographic column. The top and bottom products are withdrawn from the apparatus, respectively, during the bottom and top feed. It is shown that under certain conditions the pH driven parametric pump has the capacity for removal of protein components from one product fraction and large enrichment in the other fraction. Moreover, the continuous process can be operated with a large feed throughput.

SCOPE

Parametric pumping represents a new development in separation science. It has attracted considerable attention both because of its novelty and the possibility of continuous operation in small equipment with very high separation factors. Much experimental and theoretical work has been done on thermal and pressure parametric pumping. By contrast, very little work has been done on pH parametric pumping. Included are Sabadell and Sweed (1970), Bradley (1973), Shaffer and Hamrin (1975), Busbice and Wankat (1975), and Chen et al. (1977).

The pH parapumping involves reciprocating flow of the protein mixture to be separated through a bed of ion exchanger and, simultaneously, synchronous cyclic variation of the pH. The change of pH displaces the interphase equilibrium and, in combination with the reciprocating flow, causes preferential movement of the sorbable components of the mixture towards one end of the bed, leading to a buildup of the separation from cycle to cycle.

Recent experimental results obtained by Chen et al. (1977) have shown that parametric pump has the capacity for separating a mixture of haemoglobin and albumin. The pump considered had a center feed between an enriching column and a stripping column and was operated batchwise during upflow and continuously during downflow.

In the present paper, the feed is alternately introduced at the top and bottom of the column, while the top and bottom products are withdrawn from the apparatus during the bottom and top feed, respectively. Emphasis is placed on the operating conditions necessary to achieve high separations and high product rates. The system studied is haemoglobin-albumin on CM Sepharose. Contrary to the system with the center feed for which the haemoglobin initially present at the top of the stripping section above the point of high pH liquid penetration is immobilized there permanently, no haemoglobin immobilization problem occurs for the present arrangement.

CONCLUSIONS AND SIGNIFICANCE

The pH parametric pumping is extended to protein separation. The new continuous process developed is possible to cause certain proteins in a mixture to migrate toward one end of a chromatographic column, thereby effecting separation. Experimental data are obtained for the system of haemoglobin-albumin on CM Sepharose.

The experiments show that after an initial transient, the product concentrations reach a limiting condition and remain constant as the number of cycles continues to increase. Thus, as long as the system operates, two product streams are continually withdrawn from the apparatus. This offers significant advantages over the batch pump for which no benefit results by operating additional number of cycles after limiting concentrations are reached in the reservoirs attached to each end of the column. Furthermore, the process is capable of yielding high separation factors with large feed throughput in equipment of small size without the necessity of solid phase regeneration.

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PROCESS DESCRIPTION

The parapump considered is shown in Figure 1. It consists of a column packed with an ion exchanger and reservoirs attached to each end. Initially, the column voids, and the reservoirs are filled with the mixture to be separated. Reciprocating flow within the system causes the fluid to move up and down through the apparatus. As the flow direction changes, the column pH is also changed by changing the pH of the fluid entering the column. The top reservoir is maintained at a low pH level (P_2) by an automatic titrator, while a second titrator is used to keep the bottom reservoir at a high pH level (P_1). The pump has dead volumes V_T and V_B for the top and bottom reservoirs, respectively. The flow system has four distinct stages in each cycle:

1. Flow from the top reservoir through the column to the bottom reservoir for time t_I.
2. Feed at the bottom with the mixture of $p\text{H} = P_2$, and flow out of the top of the column as the top product for time t_{II}.
3. Flow from the bottom reservoir through the column to the top reservoir for time t_{III}.
4. Feed at the top with the mixture of $p\text{H} = P_1$, and flow out the bottom of the column as the bottom product for time t_{IV}.

The flow rate within the column is always identical to the reservoir displacement rate Q. The volumes of the bottom and top feeds (Qt_{II} and Qt_{IV}) are, respectively, equal to those of top and bottom products. For both the up and down flow, the reservoirs have the same displacement; that is, $Qt_I = Qt_{III}$.

Proteins carry both negatively and positively charged groups which can normally bound to anionic or cationic exchangers. The net charge is dependent on the pH level. At low pH, the net charge is positive. At high pH, it is negative. At the isoelectric point, that is no net charge, the proteins are not bound to any type of ion exchangers.

Figure 2 shows the schematic description of the pH parametric pumping principle. Suppose we are concerned with the separation of a two-protein system only. Let us assume that the two proteins A and B have isoelectric points I_A and I_B, whereas $I_A > I_B$. Two constant pH fields (that is, high and low pH, P_1, P_2) are imposed periodically on the system, and $P_1 > I_A > P_2 > I_B$. The ion exchanger is assumed to be cationic with counter ions S^+. For the purpose of illustration, we will make the following assumptions:

1. The displacement is equal to the void volume of the column V_e; that is, $Qt_I = Qt_{III} = V_e$.
2. The volume of either the top or the bottom feed is identical to V_e; that is, $Qt_{II} = Qt_{IV} = V_e$.
3. The ion exchanger used (CM Sepharose) has high capacity, and the ionic exchanger between the counter ions and the protein A is essentially complete at the end of each stage (Pharmacia Fine Chemicals, 1976).

At the time zero, the void volume of the bed is filled with the high pH feed solution, and the top reservoir is filled with a solution containing a feed of $p\text{H} = P_2$. Therefore, the net charges for A and B in the column are negative and in the top reservoir are positive and negative, respectively. During the first downflow stage t_I, the low pH (P_2) fluid coming from the top reservoir enters the top of the column, while the solution emerged from

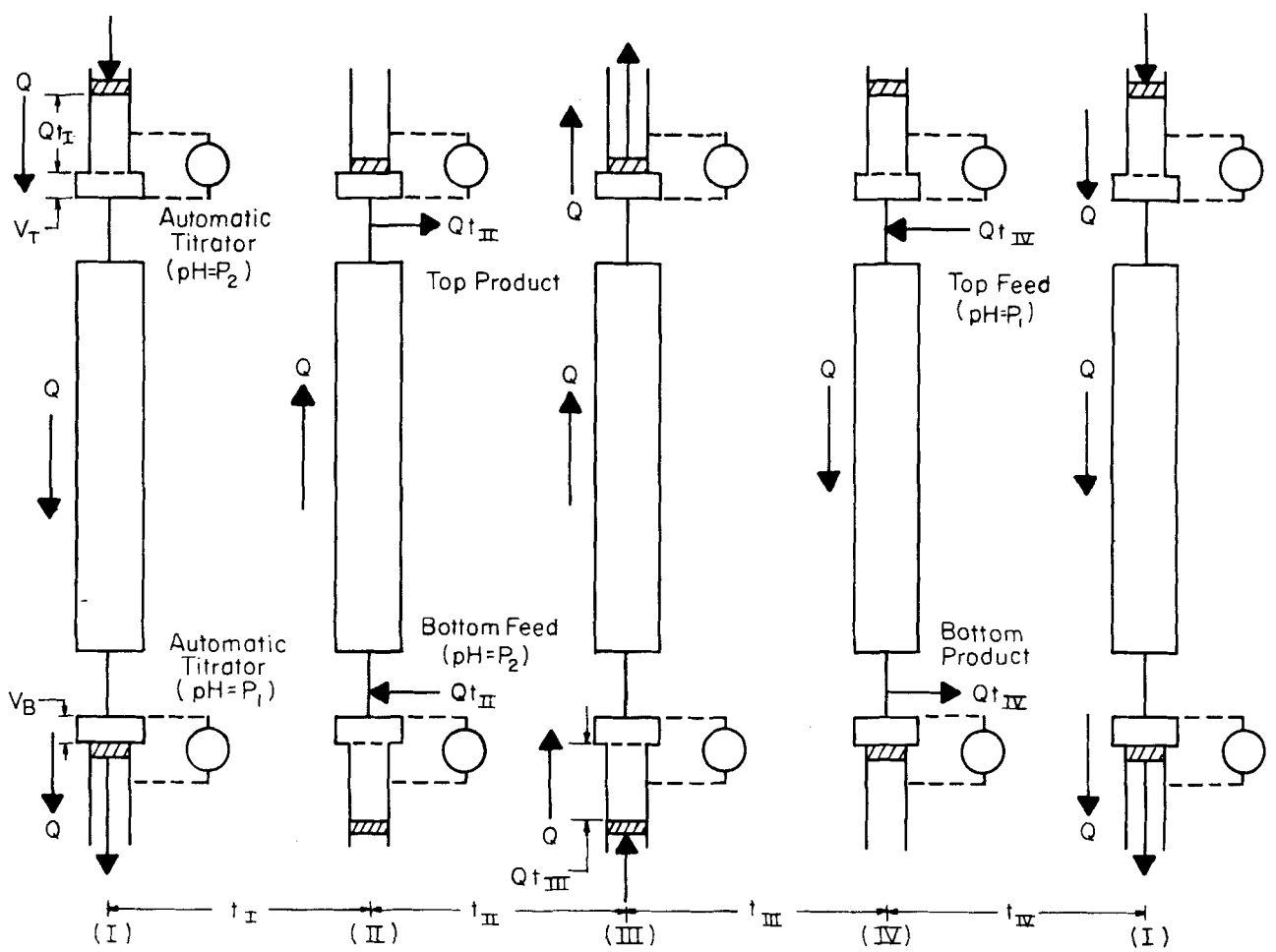

Fig. 1. Column diagram for continuous pH parametric pumping.

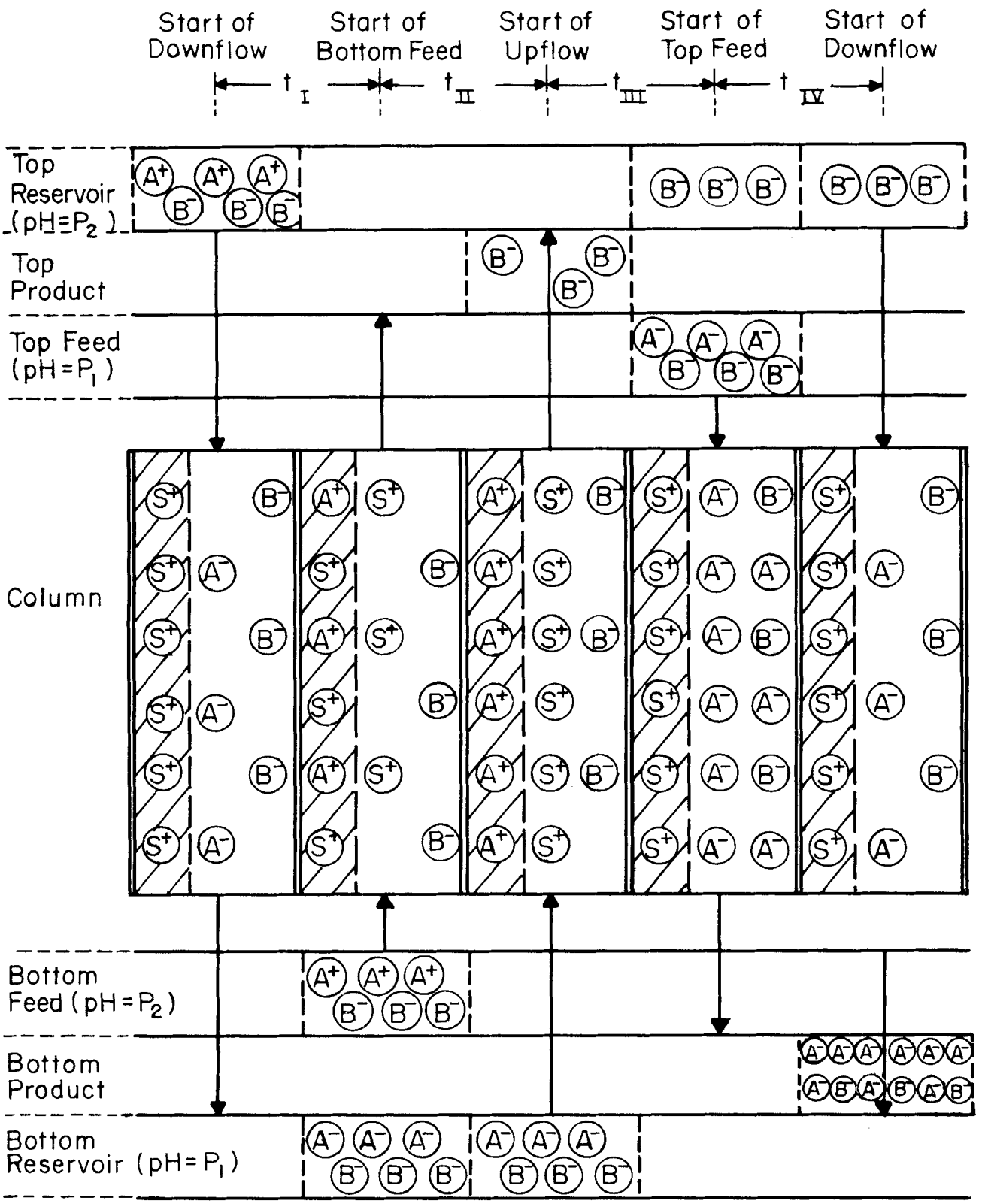

Fig. 2. Schematic description of *p*H parametric pumping principle.

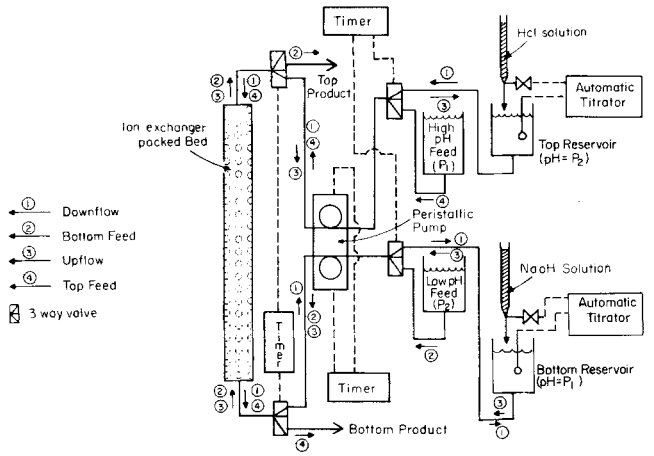

Fig. 3. Experimental apparatus for pH parametric pumping.

the other end enters the bottom reservoir. The pH of the column is then changed from P_1 to P_2. As a result, S^+ are exchanged for the A^+ originated from the top reservoir. During t_{II}, a feed with $p\text{H} = P_2$ is introduced through the bottom, and simultaneously a top product containing only pure B is removed from the column at the same rate. In addition, the A^+ initially present in the bottom feed are exchanged for S^+. After this adsorption process, an upflow is followed, and the high pH fluid enters the bottom of the column. The solution containing pure B flows out of the column to the top reservoir. Consequently, the pH in the column changes from P_2 to P_1, and desorption of A occurs. S^+ shifts back to the bed, and the ion exchanger is then regenerated. During t_{IV}, a feed with $p\text{H} = P_1$ enters the top, while a product rich in A is withdrawn from the bottom of the column. One whole cycle is thus completed. From Figure 2, one can see that all of the solute A entering from either the top or bottom always moves toward the bottom product stream. Complete removal of A from the top product stream is achieved with one single complete cycle. Note that this result is based on the assumptions made above. In practice, it may not be possible to implement the operating conditions that satisfy the required assumptions. However, an optimum separation is attainable by repeating the process, illustrated in Figure 2, in succeeding cycles. In the limit of a large number of cycles, the system is capable of removing substantially all of the A from the top product stream and transferring them to the bottom stream. The separation factor will therefore become very large.

EXPERIMENTAL

The experimental apparatus is shown schematically in Figure 3. The column (0.016 m ID and 0.4 m length) was packed with ion exchangers and maintained at a constant temperature of 288°K. Reciprocating flow within the system was introduced by a P-3 peristaltic pump manufactured by Pharmacia Fine Chemicals. The pump was connected to a dual timer to have the flow direction reversed automatically at the end of each downflow or upflow. Four automatic valves, wired to two timers, were used so that the low and high pH feeds were alternately directed to the bottom and top of the column. At the same time, the top and bottom products were withdrawn from the column, respectively, during the bottom and top feeds.

The pH levels in the reservoirs were maintained constant by titrating with hydrochloric acid and sodium hydroxide solutions. The strength of the acid and base were so chosen that the effects on the product and reservoir concentrations were minimal. To ensure perfect mixing with the titrant in the reservoirs, magnetic stirrers were used.

A haemoglobin and albumin mixture was selected to examine experimentally the feasibility of this parametric pumping separation scheme. Worthington human haemoglobin and human serum albumin were used. The isoelectric points for haemoglobin and albumin are 6.7 and 4.7, respectively. For all runs, $P_1 = 8$ and $P_2 = 6$, so that only the isoelectric point of haemoglobin lies between the two pH levels. This will lead to the result that haemoglobin would be removed from the top product stream and concentrated in the bottom product stream.

For the solid phase CM Sepharose (Registrated Trademark) ion exchange media manufactured by Pharmacia Fine Chemical was chosen. CM Sepharose is a macroporous, bead formed ion exchanger derived from the cross linked agarose gel Sepharose CL-6B. The ion exchange capacity of this material is high, and in addition the exchanger has an extremely stable bed volume.

Samples taken from the product streams at the end of each cycle were analyzed by using the spectrophotometer (Bausch & Lomb Spectronic System 400-3). The haemoglobin concentration was determined directly from the absorbance at a wavelength of 403 μm. The Bio-Rad Protein assay was used to obtain the total protein concentration. Hence, subtraction of the haemoglobin concentration from the total gave the concentration of albumin.

RESULTS AND DISCUSSION

All parametric pump separation experiments were carried out in the apparatus depicted in Figure 3. Table 1 summarizes all the experimental parameters, and the results are plotted in Figures 4 to 10. The experimental results confirm the parapump theory described above.

TABLE 1. EXPERIMENTAL PARAMETERS

Run	Feed (wt %) Haemoglobin	Albumin	Displacement rate, Q cm³/s	Feed volume (cm³) Bottom Qt_{II}	Top Qt_{IV}	Reservoir displacement $Qt_I = Qt_{III}$ (cm³)	Ionic conc. molarity, M Bottom Buffer	NaCl	Top Buffer
1	0.02	0.02	8.33×10^{-3}	10	10	22.5	0.2	0.1	0.05
2	0.02	0.02	8.33×10^{-3}	5	10	22.5	0.2	0.1	0.05
3	0.02	0.02	8.33×10^{-3}	15	10	22.5	0.2	0.1	0.05
4	0.02	0.02	8.33×10^{-3}	20	10	22.5	0.2	0.1	0.05
5	0.02	0.02	3.33×10^{-3}	10	10	22.5	0.2	0.1	0.05
6	0.02	0.02	16.67×10^{-3}	10	10	22.5	0.2	0.1	0.05
7	0.02	0.02	25.00×10^{-3}	10	10	22.5	0.2	0.1	0.05
8	0.02	0.02	8.33×10^{-3}	15	10	22.5	0.05	0.65	0.05
9	0.02	0.02	8.33×10^{-3}	15	10	22.5	0.25	—	0.025
10	0.02	0.02	8.33×10^{-3}	15	10	22.5	0.05	0.25	0.05
11	0.02	0.02	16.67×10^{-3}	10	10	35	0.2	0.1	0.05
12	0.02	0.02	8.33×10^{-3}	10	10	10	0.2	0.1	0.05
13	0.01	0.01	8.33×10^{-3}	15	10	22.5	0.25	—	0.025

For all runs: column length = 0.15 m; $V_T = V_B = 10$ cm³; $P_1 = 8$ and $P_2 = 6$.

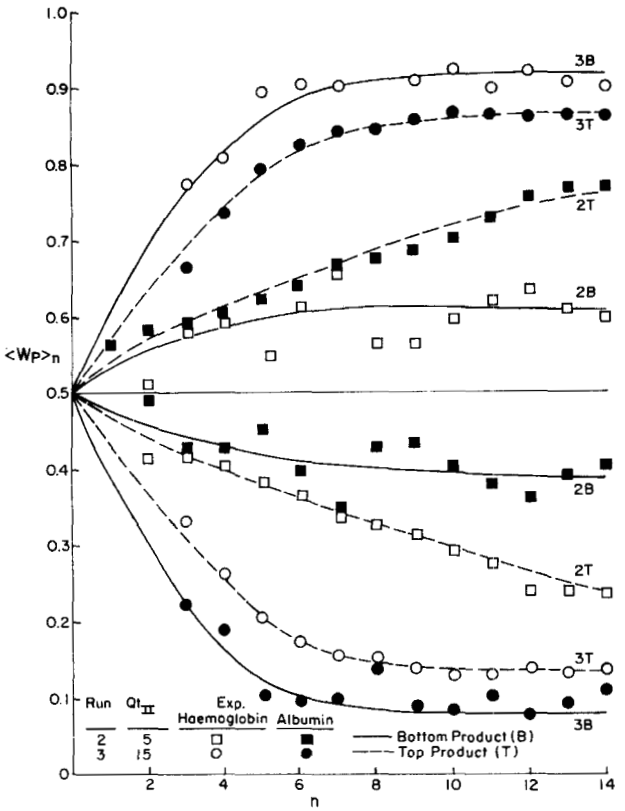

Fig. 4. Concentration transients for the top and bottom products.

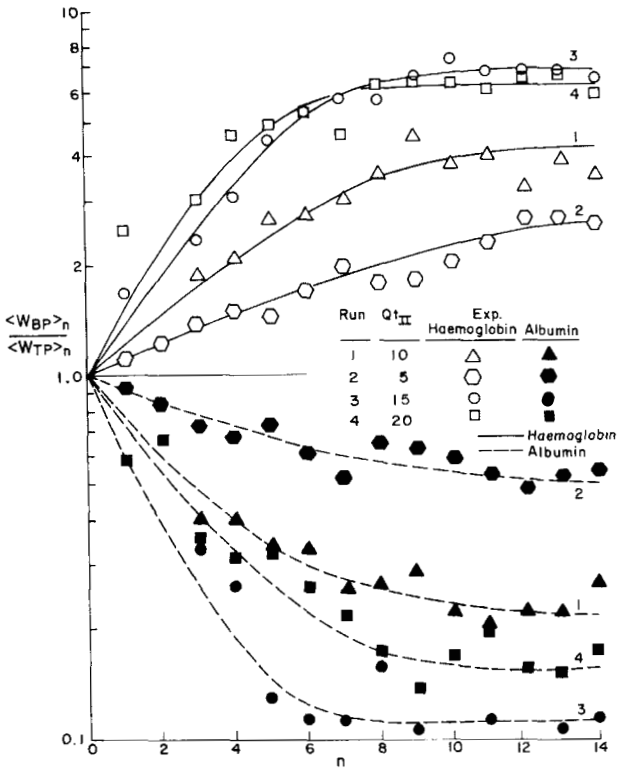

Fig. 5. Effect of Qt_{II} on separation.

Haemoglobin does migrate downward and accumulates at the bottom end.

Figure 4 shows the weight fraction of solute based on the total weight of proteins W [that is, (kilogram of haemoglobin or albumin)/(kilogram of total proteins)] as a function of n. The haemoglobin weight fraction falls in the top product stream and rises in the bottom product stream with the increasing number of cycles. The reverse holds true for albumin as observed from the same figure. As n becomes large, the weight fractions in both streams level off to steady state values.

The effect of Qt_{II} on the separation factor S.F. $\langle W_{BP}\rangle_n/\langle W_{TP}\rangle_n$, is demonstrated in Figure 5. The separation factor is defined as the quotient of the bottom and top weight fractions. One may see that for runs 1, 2, and 3, an increase in Qt_{II} produces an increase in the steady state S.F. for haemoglobin and has an opposite effect on albumin. However, if Qt_{II} becomes too excessive as in run 4, haemoglobin from the bottom feed passes through the column and out in the top product. The separation becomes worse. Note that Qt_{IV} should have the similar effect on separation as Qt_{II}.

The dependence of the separation on reservoir displacement rate Q is shown in Figure 6. From the figure, it appears that one can improve the separation by decreasing Q. However, in general practice, one would want to achieve a desired steady state separation as quickly as possible. If we plot S.F. vs. time as shown in Figure 7, we

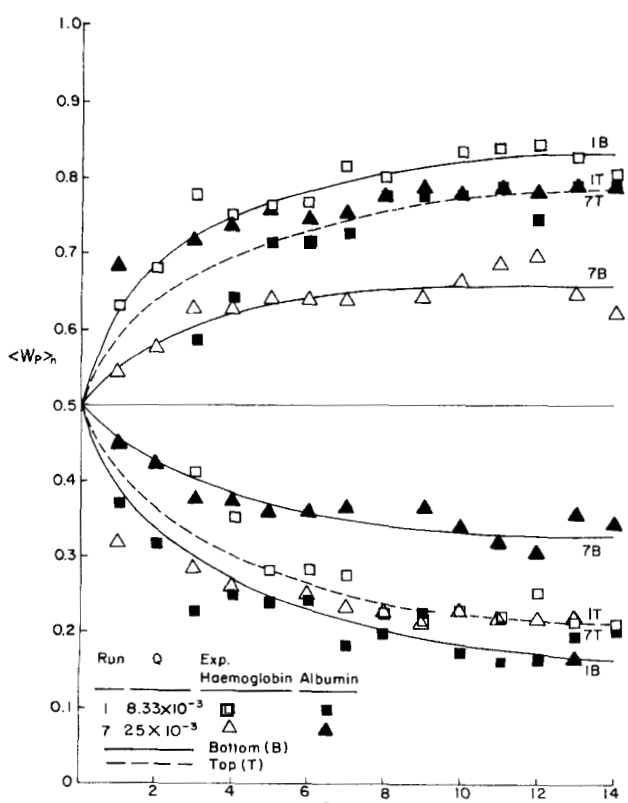

Fig. 6. Dependence of separation on the reservoir displacement rate.

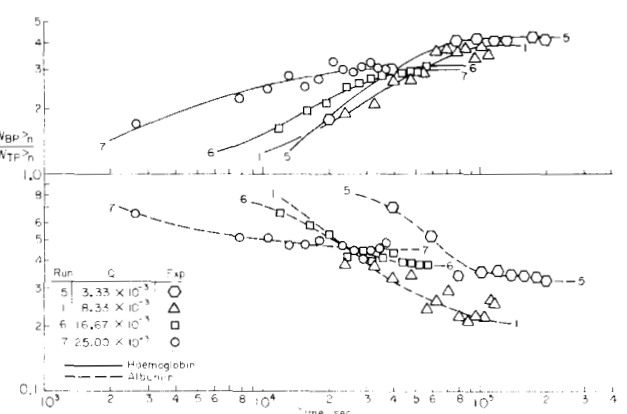

Fig. 7. Separation factors vs. time.

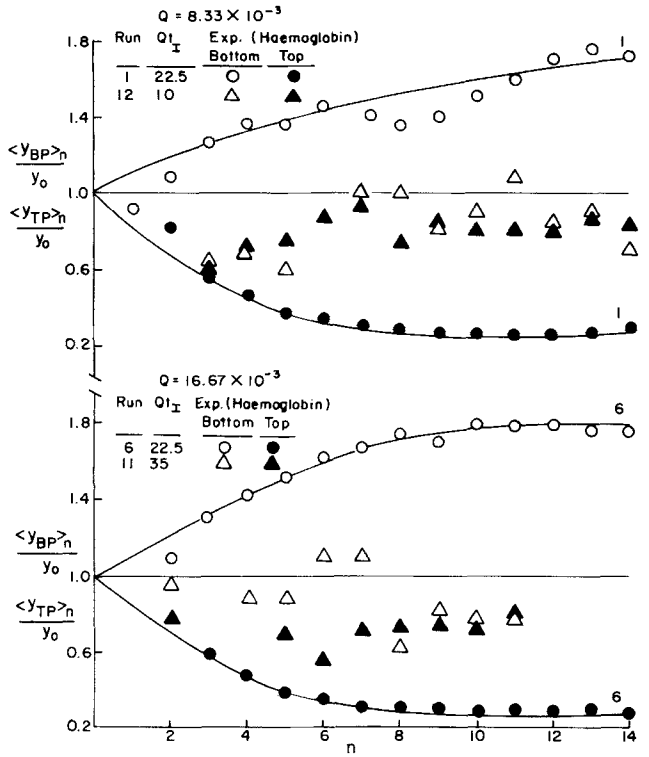

Fig. 8. Effect of reservoir displacement on separation.

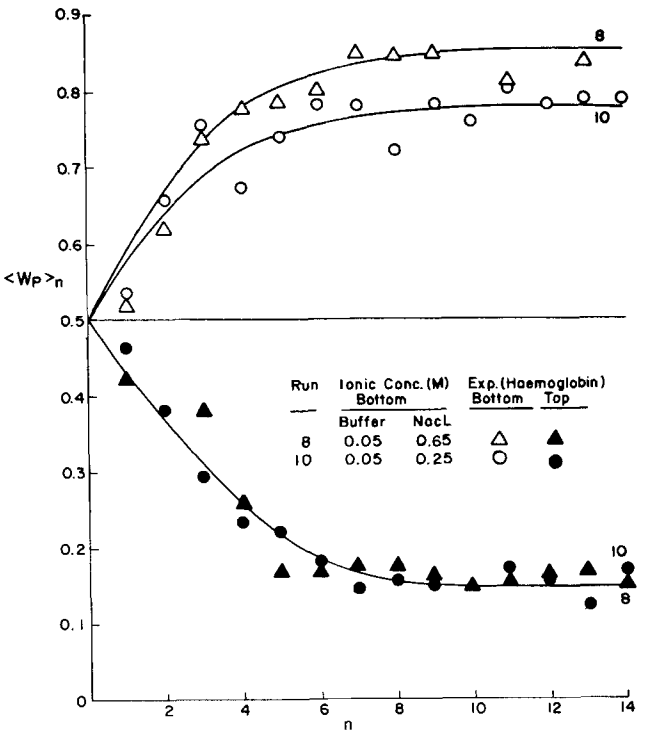

Fig. 9. Dependence of separation on ionic strength.

find that the highest Q is in fact more preferable. Smaller Q would only be used when the greater ones do not give the desirable extent of separation. Also, if Q becomes too small (run 5), axial diffusion may be important, and poor separation may result.

The effect of reservoir displacement on concentration transients is shown in Figure 8. The ordinate is the average product concentration (kilograms per cubic centimeters) divided by the feed concentration for haemoglobin. The dimensionless concentration ratios for the bottom products are always greater than one, while those for the top product are always less than one. Comparing runs 1 and 12 ($Q = 8.33 \times 10^{-3}$ cm^3/s), as well as runs 6 and 11 ($Q = 16.67 \times 10^{-3}$ cm^3/s), one can see that separation is virtually nonexistent, whereas the displacement Qt_I is much less than or greater than the column void volume V_e (22.5 cm^3). As long as Qt_I is smaller than V_e, the separation should gradually increase with Qt_I and reach an optimum value when $Qt_I = V_e$ (curves 1, 6).

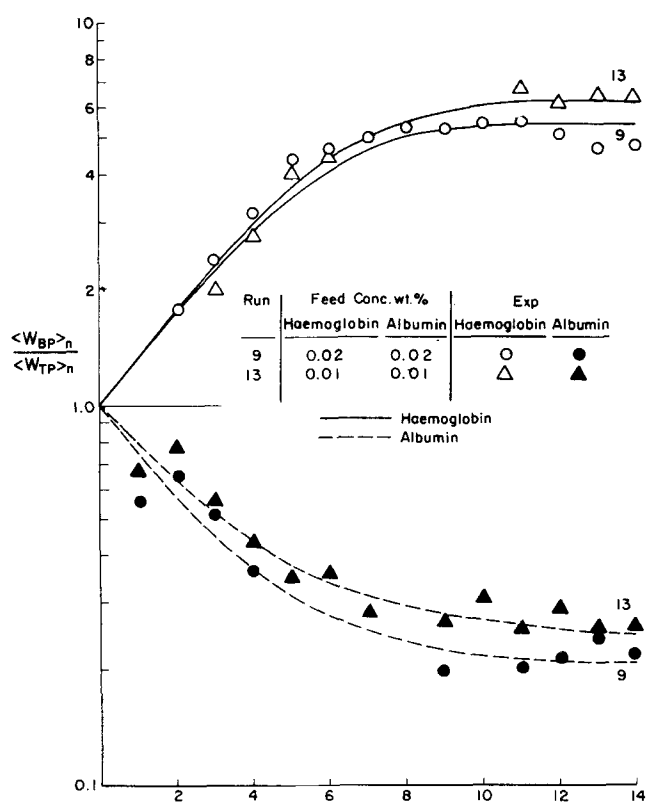

Fig. 10. Effect of feed concentration on separation.

The effect of the buffer ionic concentration on the separation is demonstrated in Figure 9. An increase in the ionic concentration (the sodium ion concentration) of the high pH solution results in an increase in the desorption of haemoglobin from the exchanger. It thus enhances the haemoglobin concentration in the bottom product, while the top concentration is essentially constant.

Figure 10 illustrates the change of the separation factor S.F. with n for two different feed concentrations. The separation factors for both cases are close to each other. Thus, the protein concentration in the feed has no significant influence on the separation. As far as the feeds are not too concentrated, the components in the mixture will not interact with one another and compete for adsorption sites on the exchangers. Under this condition, it is possible to obtain a complete separation of the two proteins by passing the product streams from one column into several other columns, placed in series.

Figure 11 shows the results of a simple experiment carried out in a column packed with a cationic exchanger (CM Sepharose). Initially, the exchanger was in equilibrium with a high pH ($P_1 = 8$) buffer. At $t = 0$, a low pH feed ($P_2 = 6$) containing 0.02 wt % of haemoglobin (upper diagram) was introduced to the top of the column, and product samples were collected from the bottom of the column in equal time intervals. Since haemoglobin at pH = 6 carried a positive charge and the ex-

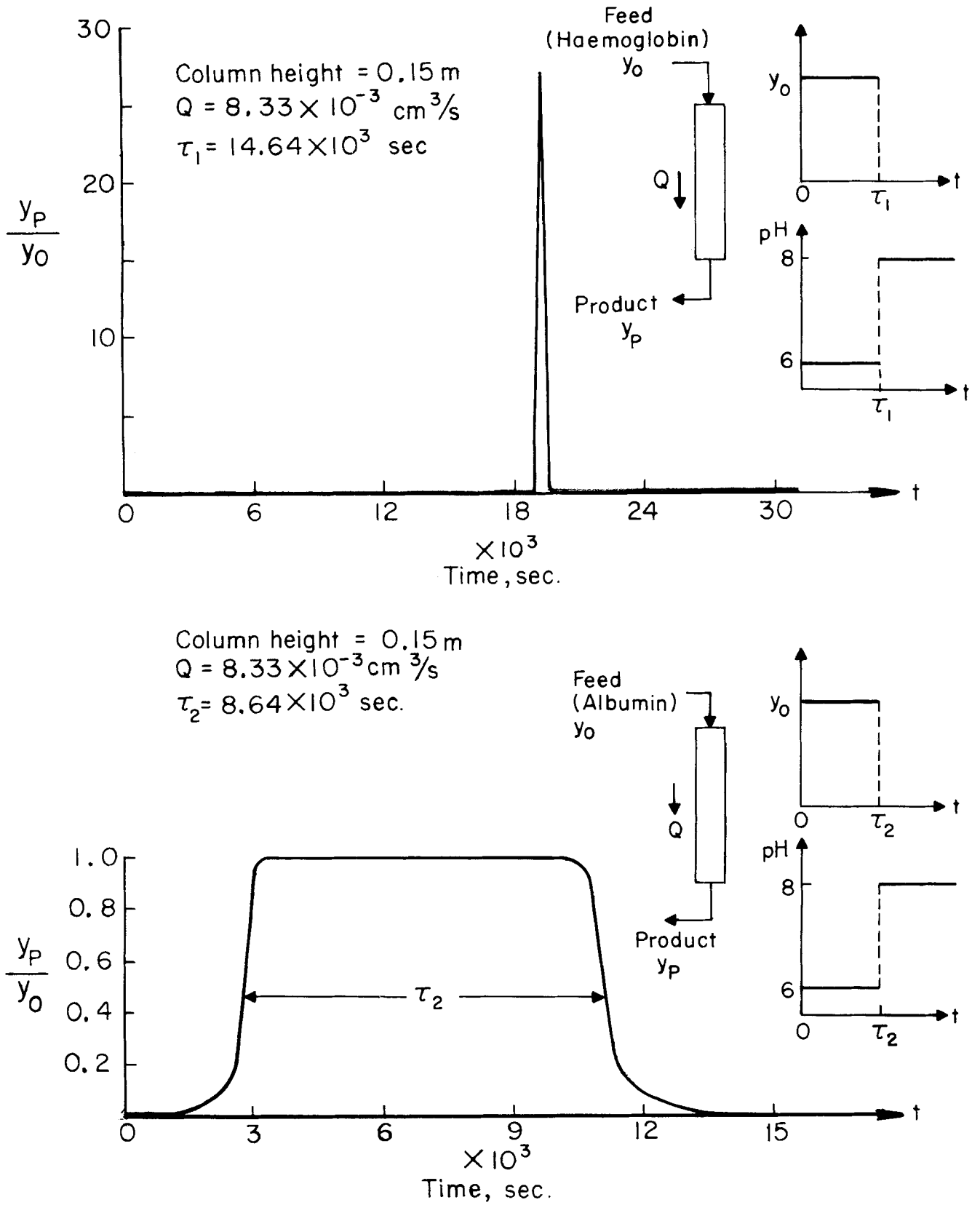

Fig. 11. Elution curves for haemoglobin and albumin.

changer had large capacity under the specific buffer concentration used, most of the haemoglobin supplied from the feed was adsorbed on exchangers. The product concentration was essentially equal to zero during the feeding period τ_1. At $t = \tau_1$, the high pH buffer ($P_1 = 8$) entered the column as elutant. Haemoglobin became negatively charged and was eluted from the ion exchanger. Hence, the exit stream concentration rose sharply to a high value and dropped to zero as soon as the haemoglobin was completely removed from the exchanger. A similar experiment was done for albumin (lower diagram). For albumin, the net charge was negative at pH = 6 or 8. The exit concentration approached a steady value (approximately equal to the feed concentration) for a time

period of τ_2 and then returned to zero. This implied that no appreciable ionic exchange took place between albumin and the exchanger.

The results shown in Figure 11 illustrate the importance of selecting the appropriate pH levels for the parapumping separation. Consider a solution of n proteins ordered according to their isoelectric point, I_i. Choose two pH values P_1 and P_2 such that

$$I_1 < I_2 < \cdots < I_m < P_2 < I_{m+1} < \cdots < I_{n-1} < I_n < P_1$$

The first m components will bear a negative charge, whereas the others will carry a negative charge at P_1 and a positive charge at P_2. Therefore, the latter group will be bound to a suitable cationic exchanger at P_2 and released at P_1, while the first m components will be unaffected. Thus, a parametric pump operating with levels of P_1 and P_2 should be capable of removing the components $m + 1, \ldots, n$ from one product stream and enriching the other product stream with these components.

Many versions of parametric pumps are conceivable. The pump examined here appears to be a very promising device for separating two or multicomponent protein mixtures.

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NOTATION

I_i = isoelectric point for component i
n = number of cycles of pump
P_1 = high pH
P_2 = low pH
Q = reservoir displacement rate, cm^3/s
Qt_I = upflow displacement, cm^3
Qt_{II} = volume of top product, cm^3
Qt_{III} = downflow displacement, cm^3
Qt_{IV} = volume of bottom product, cm^3
t_I = duration of downflow, s
t_{II} = duration of bottom feed, s
t_{III} = duration of upflow, s
t_{IV} = duration of top feed, s
V_B = bottom reservoir dead volume, cm^3
V_e = void volume, cm^3
V_T = top reservoir dead volume, cm^3
W = weight fraction of solute based on total weight of proteins, (kg/kg)
$\langle W_P \rangle_n$ = average W in the product at n^{th} cycle, (kg/kg)
$\langle W_{BP} \rangle_n$ = average W in the bottom product at n^{th} cycle, (kg/kg)
$\langle W_{TP} \rangle_n$ = average W in the top product at n^{th} cycle, (kg/kg)
y_0 = concentration of solute in the feed, kg moles/cm^3
$\langle y_{BP} \rangle_n$ = average concentration of solute in the bottom product at n^{th} cycle, kg moles/cm^3
$\langle y_{TP} \rangle_n$ = average concentration of solute in the top product at n^{th} cycle, kg moles/cm^3

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Rheological Characteristics of Solid-Liquid Mixtures

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The relationship between the shear stress and strain rate of a mixture consisting of solid particles suspended in a Newtonian fluid depends upon the physical properties of the mixture as well as kinematic features of the flow. Functional relationships for rheological properties of mixtures are derived and compared to measured results obtained by previous investigators.

SCOPE

The design of a system to transport a suspension of solids in a fluid medium requires knowledge of the constitutive equations of the mixture. A theoretical description of these equations are presented for mixtures where only mechanical and hydrodynamic effects are significant. The two-component mixture under consideration consists of rigid spherical particles suspended in a Newtonian fluid. The mixture, however, can not, in general, be considered as having Newtonian properties. Two flow regimes for a solid-liquid mixture are described, one of which does exhibit Newtonian characteristics with the other region having a nonlinear relationship between shear stress and strain rate. The theoretical analysis describing the constitutive relationships in the Newtonian range are compared to experimental results obtained by previous investigators. The analytical developments are shown to be valid for a large range of concentrations of solids.