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# SORPTION EQUILIBRIA BETWEEN PROTEINS AND CATION EXCHANGERS

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## SUMMARY

The sorption of proteins on the weak cation exchanger Amberlite CG-50 was studied and the results were interpreted in terms of a thermodynamic model involving ion-exchange equilibria and hydrogen bonds. The number of bonds between the protein and the resin, as calculated on the basis of the model, depends strongly on pH.

The distribution coefficient is an easily linearizable function of sodium ion concentration. Hence it is possible to calculate the buffer concentration suitable for equilibrium chromatography from the results of a few batch experiments.

The thermodynamic description of the ion-exchange processes provides a theoretical basis for the determination of the desired conditions for chromatography. The theory is supported by dynamic ion-exchange experiments.

## INTRODUCTION

Ion-exchange chromatography is widely used for the separation of proteins and other polyions, such as nucleic acids, from one another and to check the homogeneity of a polyelectrolyte. The dynamics of equilibrium ion-exchange chromatography, *i.e.*, when the eluent does not differ from the buffer in which the polyelectrolyte is applied to the column, can be more or less adequately described by the theoretical plate model[1], by other models, or by differential equations; these approaches have been excellently reviewed by Giddings[2]. In all of these models, the retention ratio, which is closely related to the distribution coefficient, is a parameter of crucial importance, and its value should be known. The distribution coefficient of a protein between a buffer and a given ion exchanger cannot be calculated from the amino acid composition or from the distribution coefficient of the same protein under other conditions, but must be determined experimentally in every case. In this paper, an attempt is made to correlate the distribution coefficient with the buffer ion concentration.

However, our aim was not purely practical. We have also considered the bonds between a protein and the ion exchanger, which is a question of the distribution of charged groups on the protein molecule.

We mainly carried out batch experiments, as recommended by Hirs *et al.*[3], as

a convenient method of finding the most suitable conditions for chromatography. This method has several advantages over direct chromatography: (1) it requires much smaller amounts of protein and resin; (2) it is relatively rapid; (3) the distribution coefficients can be determined over a much wider range; and (4) the results are not distorted by diffusion or non-equilibrium effects.

Most of the experiments were carried out with bovine pancreatic ribonuclease A (RNase A), which is a relatively simple protein with considerable stability, and the conditions for its equilibrium chromatography on Amberlite IRC-50 have been described[3].

## MATERIALS AND METHODS

### *Ion exchanger*

Amberlite CG-50 poly(methacrylic acid) resin was purchased from Serva (Heidelberg, G.F.R.). The resin was freed from fines by decantation, washed with sodium hydroxide solution followed by hydrochloric acid, and the procedure was repeated. The cyclized resin was dried *in vacuo* over anhydrous calcium chloride. It was stored in the $H^+$ form as a powder, and measured by its weight. Its total capacity was 9.0 mequiv./g, as determined by titration.

### *Proteins*

*Ribonuclease A.* The RNase A was separated as a fraction of bovine pancreatic ribonuclease (Reanal, Budapest, Hungary) by the method of Crestfield *et al.*[4]. RNase A was stored frozen in 0.20 *M* sodium phosphate buffer of pH 6.47. It was gel filtered before experiments in order to change the buffer.

*Aldolase.* This was prepared from rabbit muscle and recrystallized three times by the method of Taylor *et al.*[5].

*Papain.* Papain *ex Papaya* latex was a product of Koch-Light, Colnbrook, Great Britain (Batch No. 52675). Both aldolase and papain were freed from ammonium sulphate by gel filtration.

### *Activity assays*

The RNase activity was assayed by the Kunitz method[6] with yeast ribonucleic acid (Schuchardt, Munich, G.F.R.) as the substrate. The papain activity was measured with carbobenzyloxy (CBZ)-glycyl-*p*-nitrophenyl ester (Cyclo Chem., Los Angeles, Calif., U.S.A., Lot K 4900). The enzyme was activated by incubation for 1 h in 0.20 *M* sodium phosphate buffer of pH 6.2 containing 2 m*M* cysteine and 10 m*M* EDTA. The aldolase activity was assayed by the hydrazine test[7].

### *Protein concentrations*

The concentrations were determined spectrophotometrically with the aid of the following extinction coefficients:

RNase A[8]: $A_{278}^{1\%} = 7.1$

Aldolase[9]: $A_{280}^{1\%} = 7.4$

Papain[10]: $A_{278}^{1\%} = 25.0$

(recalculated on the basis of a molecular weight of 23,400 for papain).

*Determination of the sorption isotherms of proteins*

About 0.03 g of dry resin was weighed into a centrifuge tube, then stirred with 5 ml of buffer at 20° for 30 min. Phosphate buffers of pH 5.5–6.5 were used, and the sodium ion concentration varied between 0.05 and 0.35 *M*. After stirring, the suspension was centrifuged at 8000 *g* for 20 min and the supernatant discarded, and this procedure was repeated several times until the pH of the supernatant reached that of the buffer within 0.02 unit. The amount of the liquid adhering to the resin was measured by wight, then protein and buffer were added to the system and the new equilibrium was reached after stirring for 20 min. The last step was the determination of the protein concentration in the supernatant. As the protein concentration in the resin was determined in an indirect way, the experimental error was relatively large. The buffers did not dissolve UV-absorbing material from the resin in an appreciable amount. The reversibility of sorption was checked by diluting the solvent phase with buffer, and by changing the buffer medium so that it caused desorption.

*Determination of the titration curve of Amberlite CG-50*

About 0.05 g of dry resin ($H^+$ form) was weighed into a vessel and suspended in 15 ml of sodium chloride solution of the required concentration. Nitrogen was bubbled through the suspension for 15 min in order to expel carbon dioxide and then the pH was adjusted to 4.5 with 0.5 *M* sodium hydroxide solution in a pH-stat (Radiometer TTT1 c) with continuous stirring under an atmosphere of nitrogen. When the consumption of alkali ceased, the end position of the pH-stat was elevated by 0.3 pH unit. The procedure was repeated up to pH 11.1, and the titration curve was constructed from the recorder data.

## RESULTS AND DISCUSSION

*Stoichiometric description of ion exchange*

The RNase A isotherms are typical saturation curves (Fig. 1). However, they do not fit rectangular hyperbolas, which would be expected in the simplest case of uniform binding sites on the resin and a single bond between the sorbate and the resin.

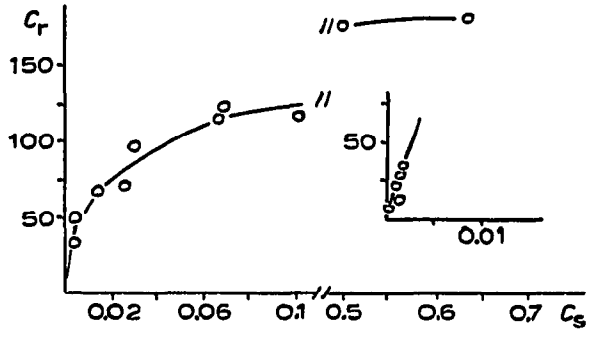


Fig. 1. Sorption isotherm of RNase A. $C_r$ = protein concentration on the resin (mg/g dry resin); $C_s$ = protein concentration in solution (mg/ml). Experimental conditions: 0.10 *M* phosphate buffer, pH 5.9, 20°. *K'* (ml/g) is the slope of the first phase. The insert shows the initial phase (the abscissa is expanded four times, the ordinate is unchanged).

Changes in sodium or hydrogen ion concentrations within one order of magnitude do not alter the shape of the isotherm, but bring about great changes in the slope of its initial, quasilinear phase. This slope ($K'$ = the amount of bound protein, mg/g resin, divided by the protein concentration in the solution, mg/ml) is the distribution coefficient of protein between a relatively dilute solution and the resin phase. In the initial phase, it is virtually independent of protein concentration. When $K'$ was small, only the initial part of the isotherm was determined.

The results can be explained quantitatively on the basis of the law of mass action. Boardman and Partridge[11] have already used this law to describe the chromatographic behaviour of cytochrome *c*. They tried to explain the dependence of elution volume on sodium ion concentration, but only qualitative agreement could be obtained in their dynamic system.

As a first approximation, we assume the formation of $n$ uniform bonds between the resin and the protein at a fixed pH, with the concomitant displacement of sodium ions bound to the resin. As the resin is not completely in the sodium form under the conditions chosen (Fig. 2), $H^+$ and $Na^+$ rearrange according to their ion-exchange equilibrium.

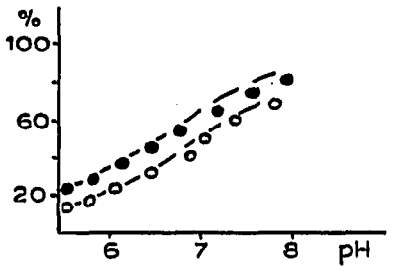


Fig. 2. Titration curve of Amberlite CG-50 resin. Percentage of titrated groups as a function of pH. Titration was carried out in the presence of 0.04 $M$ (○—○) and 0.19 $M$ (●—●) sodium chloride solution.

The equations and equilibrium constants are as follows:

$$P_s + nNa_r \overset{K_{P,Na}}{\rightleftharpoons} P_r + nNa_s \tag{1}$$

$$Na_s + H_r \overset{K_{Na,H}}{\rightleftharpoons} Na_r + H_s \tag{2}$$

where P is protein and the subscripts refer to the solution and resin-bound states. The charges are not marked. Eqn. 3 represents the net result of the above two processes, the exchange of sodium and hydrogen ions according to eqn. 2, taking place $n_2$ times:

$$P_s + n_1Na_r + n_2H_r \overset{K}{\rightleftharpoons} P_r + n_1Na_s + n_2H_s \tag{3}$$

Obviously, if $n = n_1 + n_2$, then

$$K = \frac{[P_r]\,[Na_s]^{n_1}\,[H_r]^{n_2}}{[P_s]\,[Na_r]^{n_1}\,[H_r]^{n_2}} = \frac{[P_r]\,[Na_s]^{n}}{[P_s]\,[Na_r]^{n}} \cdot K_{Na,H}^{n_2} \tag{4}$$

where the brackets refer to activities. It should be kept in mind that $K$, $n_1$ and $n_2$ depend on pH, as the protein structure may change with pH.

If the activity coefficients do not change significantly, they can be incorporated as constant factors into the values of the equilibrium constants. In general, the activity coefficients depend on ionic strength rather than on the actual concentrations of the individual ions. Therefore, we examined the changes in $K'$ in the presence of anions of different charges, while the sodium ion concentration was kept constant (Table I).

TABLE I

EXPERIMENTALLY DETERMINED DISTRIBUTION COEFFICIENTS AT DIFFERENT SODIUM ION CONCENTRATIONS AND IONIC STRENGTHS

All experiments were carried out at pH 6.23 ± 0.02 and 20°. The concentration of the buffer ion (phosphate) was 0.015 $M$.

| $Na_s^+$ (equiv./l) | Anions | $I^*$ | log $K'$ |
|---|---|---|---|
| 0.037 | phosphate + $Cl^-$ | 0.134 | 3.4 ± 0.1 |
| 0.037 | phosphate + $SO_4^{2-}$ | 0.182 | 3.5 ± 0.1 |
| 0.117 | phosphate + $Cl^-$ | 0.241 | 3.1 ± 0.1 |
| 0.117 | phosphate + $SO_4^{2-}$ | 0.361 | 2.9 ± 0.1 |
| 0.177 | phosphate + $Cl^-$ | 0.296 | 2.2 ± 0.1 |
| 0.177 | phosphate + $SO_4^{2-}$ | 0.456 | 2.1 ± 0.1 |

* Calculated: $I = 0.5\sqrt{\Sigma c_i z_i^2}$, where $c_i$ and $z_i$ are the concentration and charge of the $i$th ion, respectively.

It can be seen that the effect of ionic strength is not significant, which in turn suggests that the variations of activity coefficients can be neglected. Hence in the following discussion concentrations will be considered instead of activities.

Eqn. 4 can be transformed into

$$\log\frac{[P_r]}{[P_s]} = \log\frac{K}{K_{Na,H}^{n2}} - n\cdot\log\frac{[Na_s]}{[Na_r]} \tag{5}$$

Accordingly, if log $[P_r]/[P_s]$ is plotted *versus* log $[Na_s]/[Na_r]$, a straight line is to be expected, the slope of which is the number of bonds between the resin and the protein. The concentration is expressed as molarity in solution and mole fraction on the resin. This treatment is in accordance with Kuznetsova's reasoning[12] for small ions. At a given pH, $[Na_s]/[Na_r]$ can be varied by changing the buffer ion concentration.

We make use of the fact that pH and sodium ion concentration in the solvent phase do not change significantly when the protein expels ions from the resin, because the buffer concentration is not less than 0.015 $M$, whereas the total concentration of protein does not exceed $10^{-5}$ $M$. $[Na_r]$ is known from the titration curve of the resin; for the calculation of $[P_r]$ from the amount of bound protein, the number of functional groups accessible to the protein should be known. However, as this number is constant, it can be incorporated into the first term on the right-hand side of eqn. 5 to give

$$\log K' = \text{constant} - n\cdot\log\frac{[Na_s]}{[Na_r]} \tag{6}$$

Eqn. 6 is in good agreement with the experimental results (Figs. 3 and 5).

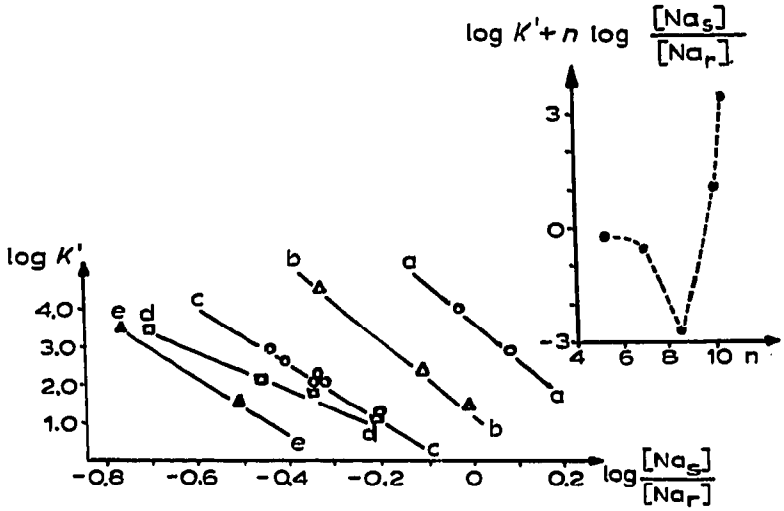


Fig. 3. Dependence of the slope of the initial phase of RNase sorption isotherm on sodium ion concentration. Conditions: 20°; pH 5.52, 5.88, 6.23, 6.47 and 6.70 for lines a, b, c, d and e, respectively. In cases a and e, linearity is assumed *a priori*. Each point is an average of 8–12 measurements. Insert: the points are the intercepts on the ordinate obtained by interpolation or extrapolation of the lines in the main figure to log $[Na_s]/[Na_r] = 0$.

*Expansion of the thermodynamic model*

The Gibbs free energy of reaction 1 is expressed by

$$-\Delta G_{P,Na} = RT \ln K_{P,Na} = RT \left( \ln \frac{[P_r]}{[P_s]} + n \ln \frac{[Na_s]}{[Na_r]} \right) \tag{7}$$

The free energy change must be proportional to the number of bonds formed, provided that they are uniform. Accordingly, log $K' + n$ log $[Na_s]/[Na_r]$ should give a straight line as a function of $n$, if the temperature is constant. However, the results contradict this expectation (Fig. 3, insert), as the deviation of the experimental points from the straight line, or even from a monotonous function, is far beyond experimental error.

This discrepancy can be solved by expanding the thermodynamic model as follows. Consider that, on sorption, hydrogen bonds are also formed, that is, the resin-bound protein exists in two different states, *i.e.*, hydrogen bonded and non-hydrogen bonded. Accordingly, the hydrogen atoms on the resin can also exist in two states, *i.e.*, interacting and non-interacting with protein. Then eqn. 6 still holds, but the ln $K' + n$ ln $[Na_s]/[Na_r]$ *versus* $n$ relationship is no longer expected to be linear. This is shown in detail in the Appendix.

*Characterization of bonds between protein and resin*

The bonds between the protein and the resin can be ionic or hydrogen linkages, and the former can be subdivided according to the ion substituted by protein.

The formation of hydrogen bonds is described by the equation

$$P_r + n_3 H_r \overset{K_H}{\rightleftharpoons} P_{r,H} \tag{8}$$

Then the negative slope of log $K'$ *versus* log $[Na_s]/[Na_r]$ is $n = n_1 + n_2 + n_3$ (*cf.* Appendix, eqn. 14).

Knowing $K_{Na,H}$, one can determine the value of $n_1$ as follows. In the absence of protein, $[H_{r,0}] = \alpha[Na_{r,0}]$ at equilibrium, provided that both the sodium and hydrogen ion concentrations in the solution are constant (the zero subscript refers to the absence of protein). Then

$$K_{Na,H} = \frac{[Na_{r,0}]\,[H_s]}{[H_{r,0}]\,[Na_s]} = \frac{[H_s]}{\alpha[Na_s]} \tag{9a}$$

If a small amount of protein is added to the system, the concentration of resin-bound ions will be decreased by $x[Na_{r,0}]$ and $y[H_{r,0}]$, respectively, but $[Na_s]$ and $[H_s]$ are virtually constant. Hence

$$K_{Na,H} = \frac{(1-x)\,[Na_{r,0}]\,[H_s]}{(1-y)\,[H_{r,0}]\,[Na_s]} = \frac{(1-x)\,[H_s]}{\alpha(1-y)\,[Na_s]} \tag{9b}$$

As can be seen, $x = y$. Considering that

$$x[Na_{r,0}] = [P_r]n_1 \tag{10a}$$

and

$$y[H_{r,0}] = y\alpha[Na_{r,0}] = (n_2 + n_3)[P_r] \tag{10b}$$

it clearly follows that $n_2 + n_3 = \alpha n_1$. Hence

$$n_1 = \frac{n}{1+\alpha} \tag{11}$$

As $n$ and $\alpha$ can be determined, $n_1$ can be calculated. The value of $n_1$ is interesting because it is the minimal number of ionic bonds between the protein and the resin. Sodium ions on the resin can be changed only by positively charged groups, whereas protons can be replaced either by positively charged or uncharged residues, and can form hydrogen bonds even between a negatively charged protein side-chain and the resin.

The values of $n$, $n_1$ and $n_2 + n_3$ for RNase A in phosphate buffers are given in Table II. It can be seen that the number of bonds changes markedly from pH 5.9 to pH 6.5, whereas the number of sodium ions replaced by one protein molecule shows much less variance. This small difference is in accordance with the fact that only about two protons are released by RNase in this pH region[13]. The number of positive charges on RNase varies between 15 and 19*, so the values of $n$ obtained by us are well within the possible range. On the other hand, the ion exchanger has a functional group every 6–7 Å according to the titration data. Thus the total interaction surface is not more than 400–500 Å, which is 6–7% of the (solvent-accessible) surface of RNase (refs. 14 and 15).

* From the amino acid composition; the range is due to the varying state of protonation of histidines. All the lysines, arginines and the amino terminal are accessible[13] in the native enzyme.

TABLE II

NUMBER OF BONDS BETWEEN RNase A AND AMBERLITE CG-50 IN PHOSPHATE BUFFERS

| *Phosphate concentration (M)* | *pH* | | | | | |
|---|---|---|---|---|---|---|
| | *5.88 (n=10)* | | *6.23 (n=7.3)* | | *6.47 (n=5.3)* | |
| | $n_1$ | $n_2+n_3$ | $n_1$ | $n_2+n_3$ | $n_1$ | $n_2+n_3$ |
| 0.05 | 2.1 | 7.9 | 2.0 | 5.3 | 1.8 | 3.5 |
| 0.10 | 2.5 | 7.5 | 2.5 | 4.8 | 2.2 | 3.1 |
| 0.15 | 3.0 | 7.0 | 3.0 | 4.3 | 2.6 | 2.7 |
| 0.20 | 3.1 | 6.9 | 3.0 | 4.3 | 2.7 | 2.6 |
| 0.25 | 3.1 | 6.9 | 3.2 | 4.1 | 2.8 | 2.5 |

Stepwise formation of bonds is not considered in the model even in its last, refined form, although no doubt it does occur. The number of bonds and the groups on the protein involved certainly change from molecule to molecule and also with time. Our determination gives an average, which is why $n$, $n_1$ and $n_2 + n_3$ are not integers. Bearing this in mind, we can disregard the individual equilibria, because the results of the less complicated approach, which we used, fit the measured values.

*Other proteins*

All of the above calculations are based on experiments with RNase A. We also examined how our model can be used to describe the interaction of Amberlite CG-50 with other proteins, namely rabbit muscle aldolase and papain.

With papain (without activation), essentially the same results are found as for RNase A. However, we observed certain peculiarities in the sorption isotherms of aldolase (Fig. 4): (1) equilibrium cannot be reached even in 30 min, and (2) the

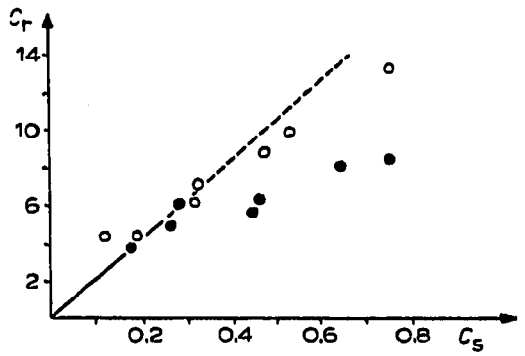


Fig. 4. Sorption isotherm of aldolase. Coordinates as in Fig. 1. Conditions: 0.15 *M* phosphate buffer, pH 6.23, 20°. Aldolase was stirred with the resin for 30 min (●—●) and 50 min (○—○).

quasilinear part of the isotherm is very short, deviation from the straight line commencing at relatively low concentrations of resin-bound protein. Moreover, the sorption is not fully reversible; 10–30 % of the sorbed protein remains bound to the resin even after stirring for 30 min with 0.35 *M* sodium phosphate buffer of pH 6.8. Never-

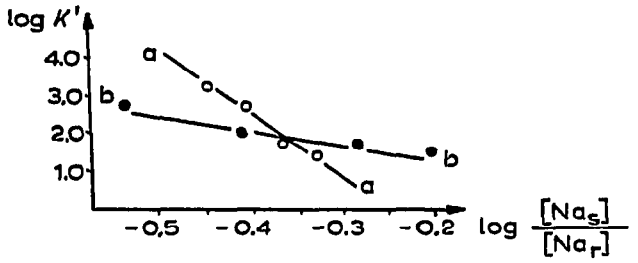


Fig. 5. Dependence of the slope of the initial phase of sorption isotherms on sodium ion concentration. (a) Aldolase in sodium phosphate buffer, pH 6.23 ± 0.05, 20°; (b) papain under the same conditions.

theless, eqn. 6 seems to be valid for both aldolase and papain (Fig. 5), which shows that our model is applicable at least to some proteins.

The anomalies observed with aldolase can be attributed to either denaturation or dissociation induced by the ion exchanger* if the different aggregational forms are bound with different equilibrium constants.

*Dynamic experiments*

It is expected that proteins can be chromatographed at equilibrium on ion exchangers, provided that their distribution coefficients are not extremely high or low. This may give practical value to the above model, because having determined the distribution coefficient ($K'$) at a given pH at two or three different buffer concentrations in batch experiments, one can then determine the composition of the buffer in which $K'$ is in the required range from the double logarithmic plot by interpolation or extrapolation.

Indeed, RNase A can be chromatographed on Amberlite CG-50 at equilibrium in 0.20 *M* sodium phosphate buffer of pH 6.47, which is the crucial step in its preparation as described by Crestfield *et al.*[4]. According to our measurements, $K'$ is about 8 ml/g under these conditions. This value can serve as a basis; proteins can be chromatographed at equilibrium if their static distribution coefficients are approximately 10. One should not expect, however, that exact peak positions can be calculated from the $K'$ values, because the error in log $K'$ is not less than $\pm 0.1$, which corresponds to a factor of 2 in $K'$, and the static distribution coefficient may differ slightly from the dynamic distribution coefficient because of non-equilibrium effects.

The following experiments supported the conclusion that equilibrium chromatography can be established according to the principles discussed above. $K'$ for RNase A in 0.20 *M* sodium phosphate buffer of pH 6.23 is about 14. It can be chromatographed at equilibrium under these conditions (Fig. 6), and separates well from RNase B.

With aldolase we failed to obtain a real equilibrium chromatogram. Even when the protein appeared just after the void volume, definite tailing and loss in activity always occurred. These phenomena could possibly be ascribed to the same reasons that caused the anomalies of the isotherm, as discussed in the preceding section.

* Rabbit muscle aldolase consists of four sub-units and does not dissociate spontaneously even in dilute solutions[16], but its dissociation can be induced by different effects[17,18].

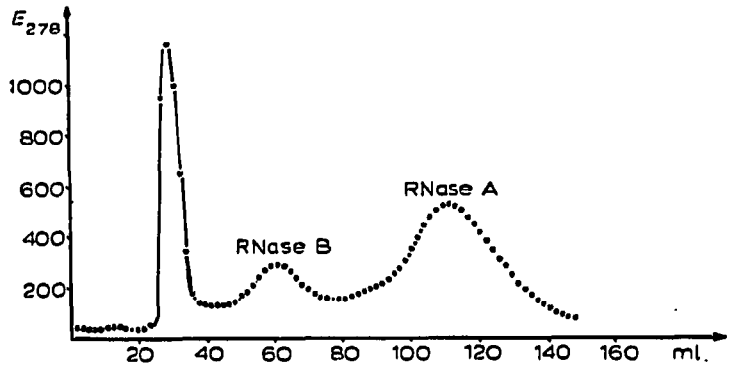


Fig. 6. Chromatography of RNase. A 60-mg amount of RNase (Reanal, without purification) in 1.6 ml of 0.20 *M* sodium phosphate buffer of pH 6.23 was applied to an Amberlite CG-50 column (1.4 × 33.5 cm) equilibrated with the same buffer and thermostatted at 20°. Flow-rate, 15 ml/h; fraction volume, 1.8 ml. The first peak is inactive.

Non-activated papain could be chromatographed in 0.20 *M* sodium phosphate of pH 6.23 (Fig. 7); *K'* was about 16. As shown by re-chromatography, it separates into three or possibly four activatable fractions. This fact may simply reflect that unactivated papain is a mixture of enzymes with different oxidation states of their essential thiol group[19,20]. Papain preparations always contain about 50% non-activatable protein. Possibly this fraction is not separated from the first two activatable peaks, which may be why the third peak apparently has greater specific activity than the preceding ones.

It is clear from the shape of the chromatogram that *K'* as determined in batch

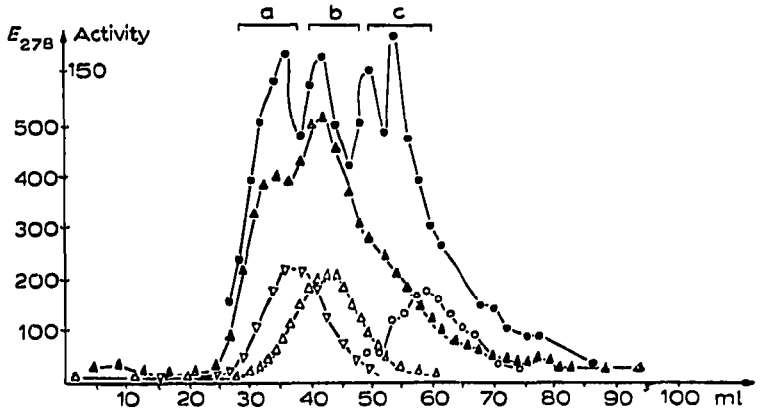


Fig. 7. Chromatography of papain. A 5.7-mg amount of unactivated papain was applied in 2.5 ml of 0.2 *M* phosphate buffer of pH 6.23 to a 1.4 × 33.5 cm (51.5 ml) Amberlite CG-50 column equilibrated with the same buffer. The column was thermostatted at 20°. Fractions of 2.0 ml were collected and analyzed for absorbance and enzyme activity. Flow-rate: 12 ml/h. ▲, Optical density; ●, activity. The main fractions were combined, as marked by brackets, and re-chromatographed. Re-chromatograms: a, △, and b, △, optical density; c, ○, activity.

experiments is a weighted average of at least three, not very different, distribution coefficients.

The results with RNase A and papain support the practical usefulness of our approach.

## APPENDIX

The partial reactions considered in the thermodynamic model of protein sorption are as follows (with the corresponding equilibrium constants):

$$P_s + n'Na_r \overset{K_{P,Na}}{\rightleftharpoons} P_r + n'Na_s \tag{1a}$$

$$Na_s + H_r \overset{K_{Na,H}}{\rightleftharpoons} Na_r + H_s \tag{2}$$

$$P_r + n_3H_r \overset{K_H}{\rightleftharpoons} P_{r,H} \tag{8}$$

The sum of the simultaneous equilibria is eqn. 2 taken $n_2$ times, *i.e.*

$$P_s + n_1Na_r + n_2H_r + n_3H_r \overset{K}{\rightleftharpoons} P_{r,H} + n_1Na_s + n_2H_s \tag{12}$$

where $n' = n_1 + n_2$ and $n = n_1 + n_2 + n_3$.

The resin-bound protein is distributed between two different states:

$$[P_{r,T}] = [P_r] + [P_{r,H}] = [P_r](1 + K_H[H_r]^{n_3}) = [P_{r,H}](1 + 1/K_H[H_r]^{n_3}) \tag{13}$$

Taking into account

$$\frac{K}{K_{Na,H}^{n_2+n_3}} = \frac{[P_{r,H}][Na_s]^n}{[P_s][Na_r]^n[H_s]^{n_3}} \tag{14}$$

which is a consequence of the definition of equilibrium constants, it follows that

$$\frac{[P_{r,T}][Na_s]^n}{[P_s][Na_r]} = \frac{K[H_s]^{n_3}}{K_{Na,H}^{n_2+n_3}}(1 + 1/K_H[H_r]^{n_3}) \tag{15}$$

Although $[H_r]$ changes with $[Na_s]$ even if the pH is constant, the right-hand side of eqn. 15 can be taken to be constant, because $K[H_r]^{n_3} \gg 1$, if the hydrogen bonds involved are of the usual stability (energy of binding 5–10 kcal/mole). The logarithm of eqn. 15 has the same form as eqn. 6, so the latter is also valid for the expanded model.

On the other hand

$$-\frac{\Delta G_{P,Na}}{RT} = \ln\frac{[P_r][Na_s]^n}{[P_s][Na_r]^n} = \ln\frac{[P_{r,T}]}{[P_s]} + n\ln\frac{[Na_s]}{[Na_r]} - \ln(1 + K_H[H_r]^{n_3}) \tag{16}$$

In eqn. 16, the change in the third term on the right-hand side is not negligible, because $[H_r]$ changes between 0.4 and 0.9 under the conditions used (Fig. 2). The non-monotonity of the $\ln [P_{r,T}]/[P_s] + n \ln [Na_s]/[Na_r]$ *versus* $n$ relationship is understandable, as $n_3$ is not necessarily a monotonous function of $n$. In fact, $n_3$ depends much more upon the juxtaposition of the proper functional groups (that is, on alterations in protein structure) than on the actual concentration of hydrogen ions in the resin, provided that the latter is not too small.

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