Decrease of tailing of chromatographic peaks

It is often found that whereas excellent chromatographic separations can be achieved with small quantities of substances, separation deteriorates badly if the load is increased. The usual reason for this phenomenon of overloading is that the partition coefficient of the substance between the mobile and stationary phases changes with its concentration. This is because the presence of other molecules of the same substance affects the partition of any given molecule between the two phases.

An unusually clear-cut case is the partition chromatography of substances with charged molecules on ion exchange resins, because here the partition coefficients depend largely on two factors. The first is the charge on the solute molecule, itself often determined by the pH of the medium. The second is the competition for the sites of the resin that the molecules of solute suffer. Hence the commonest cause of tailing of a peak on a resin is that the competition suffered by each molecule of the substance being chromatographed is appreciably higher in the peak, because of the presence of other charged molecules of the same substances, than at the edges where only the ions of the eluent solution are present. The peak therefore catches up its leading edge, and leaves a tail behind. This is particularly likely when a multivalent substance is being chromatographed with univalent ions in the eluent, because the effective competition may be appreciably raised in the peak, even though the total concentration of ions is not. The use of an eluent strong in both buffering power and concentration of competing ions usually minimizes such effects.

An exception to this was found on attempting to chromatograph 2-hydroxymethyl-3-hydroxypyridine on a sulphonated polystyrene resin. Very bad tailing of the peaks was observed when the crude product of hydroxymethylating 3-hydroxypyridine by the method of URBANSKI[1] was chromatographed in sodium acetate buffer. The pH of the effluent fractions was measured and found to be constant within 0.02 unit. The load was quite low and the concentration of competing sodium ion 0.5 N, so the usual explanations were inapplicable. It was therefore argued that other molecules of the solute might be more effective competitors than sodium ions. They might possess a greater affinity for the resin phase than that due to ionic attraction, because their aromatic rings could also interact with those of the resin. Chromatography was therefore tried in a buffer of pyridine acetate, in the hope that pyridinium ions would compete more equally with the solute ions. This greatly diminished the tailing as shown in Fig. 1.

Experimental and results

Chromatography. Fig. 1a shows the chromatography of the mixture of products in the buffer of 0.1 M acetic acid and 0.5 M sodium acetate. The skew shape of the peaks is clear. Fig. 1b shows chromatography in a buffer of 0.6 M acetic acid and 0.6 M pyridine. The peaks are much sharper. Since this solution had a pH of 5.0, and the pK's of acetic acid and pyridine are 4.7 and 5.3, this solution is expected to have concentration of 0.2 M of the neutral molecules of pyridine and acetic acid, and concentrations of 0.4 M of pyridinium and acetate ions. Even though the pH was lower than in Fig. 1a (5.0 as opposed to 5.4), which should give the solutes a greater positive charge and so retard them further, and the concentration of competing cations is lower (0.4 M as opposed to 0.5 M) also favouring retardation, the peak is

much further forward. This confirms the idea that pyridinium ions are competing with the solutes much more favourably than sodium ions. Fig. 1c shows the result of modifying the sodium acetate buffer to one (0.6 *M* acetic acid and 1.2 *M* sodium acetate) with a concentration of sodium ions high enough to elute the peaks as far forward as in the pyridine–acetic buffer. It is interesting that the peak is still wider in the sodium-ion solution, even though the competition at this sodium-ion concentration must be as high as in pyridine–acetic solution since the peak is as far forward.

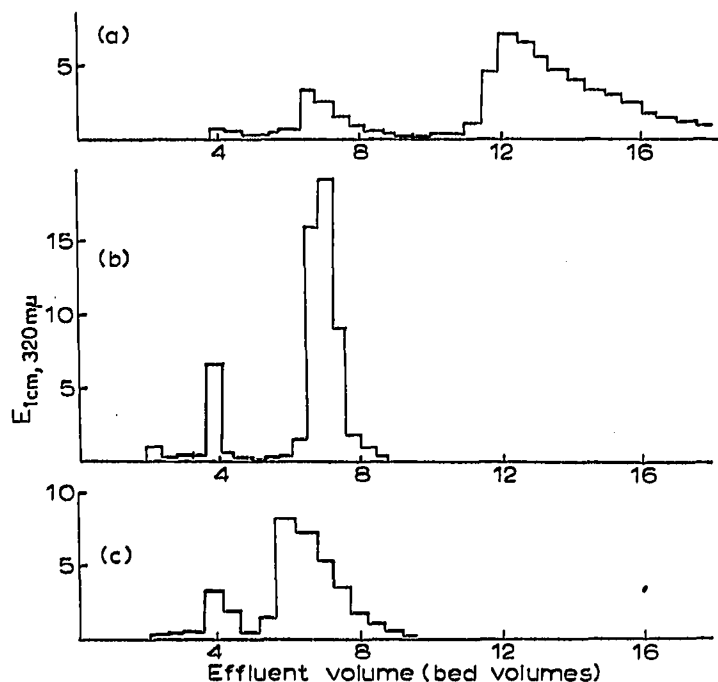

Fig. 1. Chromatograms of pyridine derivatives. A sample of 50 mg of the hydrochlorides of the products of hydroxymethylating 3-hydroxypyridine was applied to the column of 26.2 cm × 0.825 sq. cm sulphonated polystyrene beads of 8% cross linking of under 200 mesh (Zeo-Karb 225, SRC 16), which had been equilibrated, and was eluted at 30 ml cm^{-2} h^{-1}, with one of the following solutions: (*a*) 0.1 *M* acetic acid, 0.5 *M* sodium acetate; (*b*) 0.6 *M* acetic acid, 0.6 *M* pyridine (pH 5.0); (*c*) 0.6 *M* acetic acid, 1.2 *M* sodium acetate (pH 5.0).

Evidently the competition exerted by pyridinium ions is of a different kind. Although the same load was applied to each column the peak was over twice as high in the pyridine–acetic solution. In this solution the column exhibited a resolution of 10 plates per cm calculated by the method of STACK-DUNNE (cited by DIXON[2]). This calculation cannot strictly be applied to skew peaks, but a rough calculation gives about 2 plates/cm for Fig. 1c.

Identification of the substances. On two occasions equal amounts of the two peaks were obtained on treating 3-hydroxypyridine with formaldehyde by URBANSKI's method[1]. A solution of the mixed hydrochlorides (5 g in 100 ml) was run through a column of 20 cm × 3 cm diameter of polystyrene beads, 100–200 mesh, containing quaternary ammonium groups (De-Acidite FF, SRA 71) in the free base form, and displaced with 0.05 *N* HCl at a flow rate of 1.5 ml cm^{-2} min^{-1} (*cf.* PARTRIDGE AND BRIMLEY[3]). Fractions were collected and analysed by paper electrophoresis of 10 μl of each in a volatile buffer at pH 4.4 for 10 min at 80 V/cm in the system of GRAY AND HARTLEY[4]. The hydroxypyridines were detected by the blue fluores-

cence that samples of over 50 μg showed on the dried paper. On the basis of the electrophoretic results the fractions were pooled, acidified with hydrochloric acid, taken to dryness on a rotary evaporator and the hydrochlorides recrystallized by the method of URBANSKI[1] by adding acetone to an aqueous solution. The first material displaced corresponded to the second peak in Fig. 1. On titration of 100 mg of the hydrochloride with 0.1 N NaOH it showed pK's of 4.8 and 9.0 and an equivalent weight of 164 ($C_6H_8ClNO_2$ 161.5). This is consistent with its being 2-hydroxymethyl-3-hydroxypyridine, as is the fact that it was the major product in the preparation whose chromatography is shown in Fig. 1, and this was URBANSKI's[1] product. This is supported by the fact that crystallization by his method of the fractions that showed both compounds yielded this same product. The last fractions, containing the substance corresponding with the first peak in Fig. 1, gave a hydrochloride which on titration exhibited pK's of 4.5 and 8.5 and an equivalent weight of 196, and on analysis showed C 44.6% H 5.0% ($C_7H_{10}ClNO_3$ 191.5, C 43.8%, H 5.2%). This is therefore consistent with its being a di-(hydroxymethyl)-3-hydroxypyridine. WILLIAMS[5] identifies it by n.m.r. as 2,6-di-(hydroxymethyl)-3-hydroxypyridine, and confirms that the main compound is 2-hydroxymethyl-3-hydroxypyridine. STEMPEL AND BUZZI[6] and HEINERT AND MARTELL[7] also noted the production of 2,6-di-(hydroxymethyl)-3-hydroxypyridine in URBANSKI's[1] method.

Discussion

Ionic forms of the solutes. The pK's found suggest that these compounds have similar acid-base properties to the 3-hydroxypyridines studies by METZLER AND SNELL[8]. The pK's of 4–5 are therefore probably due to ionization of both the phenolic hydroxyl and the nitrogen-bound proton, with the former predominating. The compounds therefore possessed significant fractions of their molecules in each of the three forms of cation, zwitterion and uncharged molecule under the chromatographic conditions used.

Non-ionic interactions. Although it is clearly unnecessary to call attention to the possibility of specificity in competition for adsorbing sites in fields like enzymology, this possibility may be overlooked with ion exchange resins. Many factors, other than purely ionic ones, will determine the partition of solutes between resin gel and aqueous phases. Unfortunately a custom has grown up of classifying ion-exchange chromatography as a different technique from partition chromatography rather than an example of it. This classification underemphasizes the role of non-ionic interactions in separations on ion-exchange resins. Many of these are important. Thus MOORE AND STEIN[9] pointed out that their separation of amino acids on a sulphonic resin depended on non-ionic as well as ionic interactions. The elution order of glycine, alanine, valine and leucine suggests hydrophobic bonding with the resin phase. They noted that addition of propanol to the eluent selectively accelerated the less polar amino acids. KRESSMAN AND KITCHENER[10] had already pointed out the increased affinity alkyl groups gave ammonium ions for sulphonic resins. SAMSONOV and co-workers (*e.g.* references 11–12) have studied the auxiliary bonds in detail, especially from proton–solute exchange and have measured entropy and enthalpy contributions. DMITRYENKO AND HALE[13] have shown co-operative effects in proton-chlorotetracycline exchange, another departure from ideal behaviour which might be eliminated by using exchange with a more similar substance.

LEDERER AND OSSICINI[14] have clarified the role of salting out in determining the rate at which many ions chromatograph on ion-exchange papers.

DIXON[15] observed change of the partition coefficient of a peptide between a sulphonated polystyrene and an aqueous solution when urea was added to the solution, with the coefficient being altered in favour of the solution. The present example shows that a change in structure of the competing ion may be important, although the nature of the resin phase is also thereby changed.

The tailing of peaks of hydroxypyridine derivatives was greatly decreased when pyridinium replaced sodium cations in competing with these solutes for the sulphonate groups of a resin. This is attributed to a high affinity of pyridinium ions and their derivatives for the resin due to non-ionic interactions. When sodium was the competing cation it would have been impossible to maintain a constant competition as the solute concentration varied.

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