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Systematic optimization of protein separations on high-performance ion-exchange chromatographic media

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ABSTRACT

The options for improving ion-exchange separations of proteins by manipulation of operating conditions on a single column were systematically examined. Manipulation of the slope of the ionic strength gradient involved a compromise between resolution and detection. Improved resolution without loss of sensitivity was obtained by manipulating selectivity. The manipulation of relative retentions by pH adjustment was shown for both anion and cation exchange. Changes in selectivity associated with alternative buffer ions at constant pH were documented. Improvements were also observed with changes in the counterion, and temperature had a direct impact on selectivity. The systematic optimization of these variables was facilitated by incorporation into the Auto-Blend™ Method. The application of this approach to the partial purification of mouse liver lactate dehydrogenase was tested.

INTRODUCTION

Significant improvements in chromatographic protein separations are observed when rigid high-performance anion- and cation-exchange packing materials are used with appropriate instrumentation in place of traditional large particle, compressible packings. The modern, resin-based materials give excellent recoveries of biological activity. In addition, the speed and reproducibility of this chromatography greatly facilitates optimization of the operating conditions. A systematic approach to the several parameters that influence protein separations is required to exploit this potential. Using an automated system, the effect of buffer composition, eluent pH, counter ion species, gradient duration and temperature on the resolution of protein standards will be demonstrated. Adjustments of these parameters is shown to influence the purity and the recovery of an active enzyme from a crude extract of mouse liver. The principles demonstrated in this work can be applied to the optimization of protein separations by ion exchange chromatography.

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EXPERIMENTAL

Chromatography of protein standards was performed on a Waters 650 advanced protein purification system, a refrigerated WISP 712 autosampler, a 484 tunable UV detector at 280 nm, and a 820 data station. Chromatography of mouse liver extracts was performed on a Waters W600 multi-solvent delivery system, a U6K manual injector, a M490 multi-wavelength detector at 280 nm, and a 840 chromatography control and data station.

The Protein-Pak™ columns were from Waters and include DEAE 8HR (100 × 10 mm), SP 8HR (100 × 10 mm), and DEAE 5PW (75 × 7.5 mm). All buffer components and reagents were the highest available commercial grades. Standard proteins were purchased from Sigma (St. Louis, MO, U.S.A.) and were repurified to give a single major peak by ion-exchange chromatography. The standards included hen conalbumin (C-0755), bovine α-chymotrypsinogen (C-4879), horse cytochrome *c* (prepared with TCA; C-2506), hen lysozyme (L7001), hen ovalbumin (A-5503), bovine ribonuclease A (R-5000), human transferrin (T-4515), and soybean trypsin inhibitor (T-9003). Mouse liver extract was prepared by homogenizing fresh mouse liver in 0.02 *M* Tris–HCl, pH 7.5, 5 ml/g, and centrifuging at 48 000 *g* at 4°C for 30 min. The extracts (150 μl/analysis) were chromatographed without further treatment. Lactate dehydrogenase activity was estimated colorimetrically by mixing 100 μl of each fraction with 500 μl of a reaction mixture including 10 mg/ml nicotinamide adenine dinucleotide, 1 mg/ml phenazine methosulfate, 10 mg/ml nitroblue tetrazolium, and 48 mg/ml lithium lactate in 0.1 *M* Tris–HCl, pH 7.5.

RESULTS AND DISCUSSION

Protein separations on ion-exchange media are commonly optimized by adjusting the ionic strength gradient such that the increase is more gradual over the range that the protein of interest is eluted. The power and limitations of the approach were tested over the range of 0.10 *M*/column volume to 0.0125 *M*/column volume. The retention of the protein standards is plotted as a function of gradient duration in Fig. 1, showing that the peaks are further apart with more shallow gradients. The chromatograms at the extreme ends of this test series are compared in Fig. 2. The improved resolution is associated with reduced peak height. When the volume of the

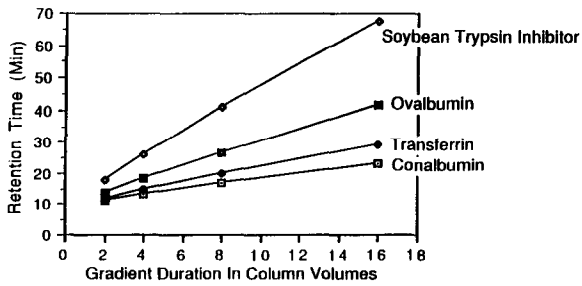

Fig. 1. Retention as a function of peak volume. Protein standards were chromatographed on a Waters Protein-Pak DEAE 8HR (100 × 10 mm) in 0.02 *M* Tris–HCl at pH 7.5. The proteins were eluted with a gradient 0 to 0.2 *M* NaCl over 2, 4, 8 and 16 column volumes.

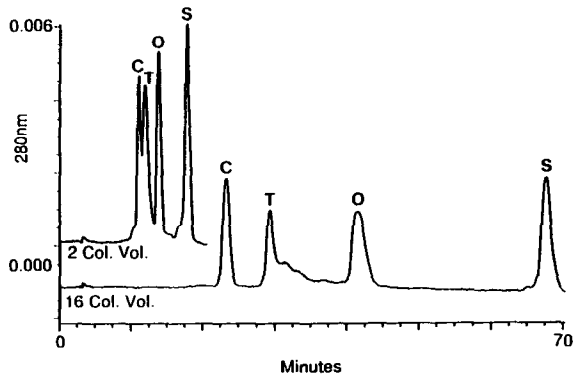

Fig. 2. Effect of gradient duration on resolution. The chromatography of protein standards with a 2- and 16-column volume (Col. Vol.) gradient is compared. Conditions as for Fig. 1. C = Conalbumin; T = transferrin; O = ovalbumin; S = soybean trypsin inhibitor.

ovalbumin peak is plotted as a function of gradient slope (Fig. 3), the increase in sample dilution with more shallow gradients is apparent. With lower protein concentration the sensitivity of UV detection is reduced, and there will be a similar effect on any enzymic or bioassay. In addition, the recovery of biological activity may be compromised by protein denaturation at high dilution. To improve protein separations without the adverse effects associated with extremely shallow gradients, those parameters that control the selectivity of the separation must be exploited. Such options would include a variety of chemical changes, but buffer pH, ionic composition of the buffer, counterion, and temperature are most amenable to systematic optimization.

The retention of protein standards as a function of pH in anion exchange chromatography in shown in Fig. 4. The retentions do change to a different extent for each protein in the mixture as is consistent with modification of chromatographic selectivity. As shown in Fig. 5, the chromatograms reflect significant differences in resolution. Conalbumin and transferrin are better resolved at pH 7.0 than at pH 9.0, while the retention of ovalbumin is virtually unaffected over this pH range. This

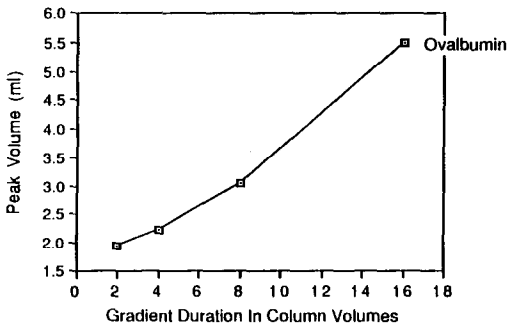

Fig. 3. Effect of gradient duration of peak volume. The volume of the ovalbumin peak when chromatographed as in Figs. 1 and 2 is shown as a function of gradient duration.

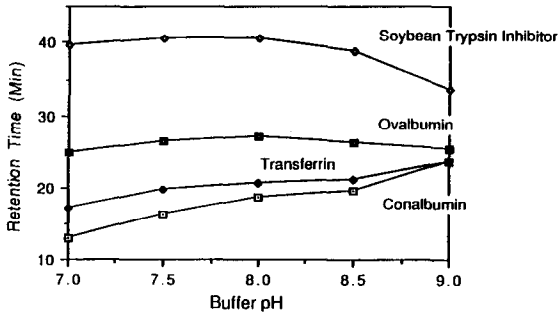

Fig. 4. Retention as a function of buffer pH. A series of protein standards was chromatographed on a Protein-Pak DEAE 8HR (100 × 10 mm) in 0.02 *M* Tris–HCl at selected pH values between 7 and 9. The same ionic strength gradient from 0 to 0.2 *M* NaCl over 8 column volumes was used at each pH.

experiment suggests that it is desirable to optimize empirically the pH to be used for the separation of a particular protein from a complex mixture.

Protein retention as a function of pH in cation exchange chromatography is shown in Fig. 6. Altered selectivity is again observed. The slopes of the retention

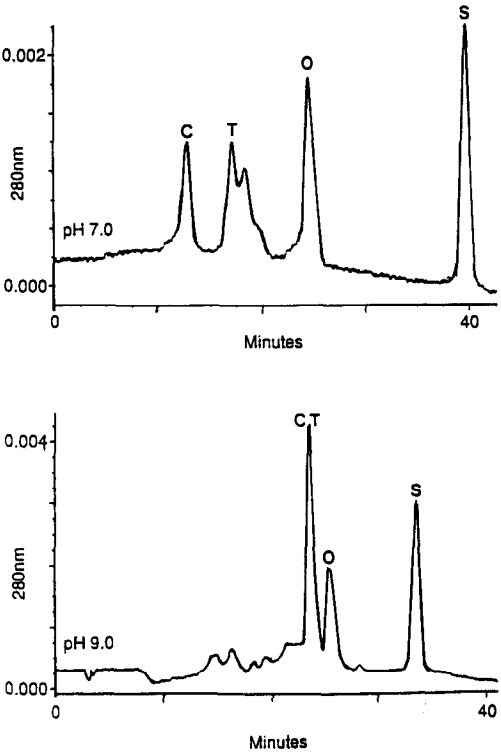

Fig. 5. Resolution as a function of buffer pH. The chromatography of protein standards at pH 7 and 9 is compared. Conditions as for Fig. 4. Abbreviations as in Fig. 2.

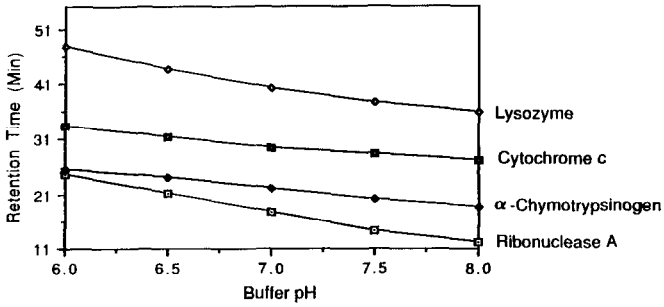

Fig. 6. Retention as a function of buffer pH. A series of protein standards was chromatographed on a Protein-Pak SP 8HR (100 × 10 mm) in 0.02 *M* phosphate at selected pH values between 6 and 8. The same ionic strength gradient from 0 to 0.3 *M* NaCl over 8 column volumes was used at each pH.

curves suggest that there may be an advantage in extending the comparison to lower pH values. Chromatography utilizing the same ionic strength gradient at pH 5.5 shows a change in selectivity compared to the separation at pH 6.0 (Fig. 7). The elution order of ribonuclease and chymotrypsinogen is reversed. The retention of

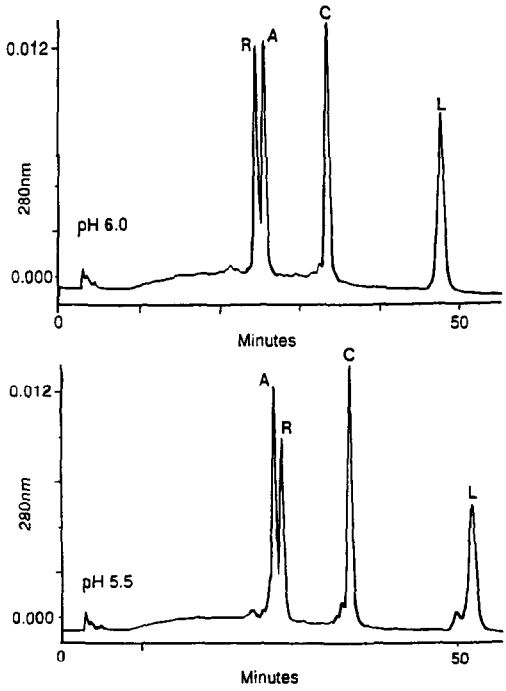

Fig. 7. Selectivity in cation exchange chromatography as a function of buffer pH. The results of cation-exchange chromatography of protein standards at pH 6.0 and 5.5 are shown. Conditions as for Fig. 6. R = Ribonuclease A, p*I* 9.5; A = α-chymotrypsinogen, p*I* 9.1; C = cytochrome *c*, p*I* 10.6; L = lysozyme, p*I* 11.0.

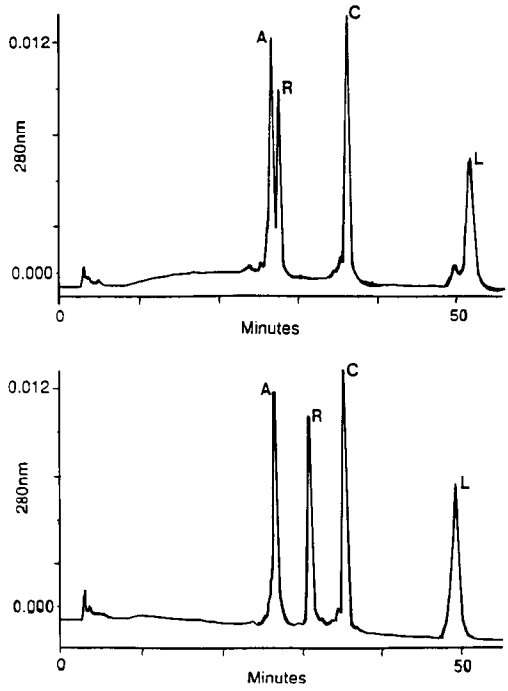

Fig. 8. Selectivity in cation-exchange chromatography as a function of buffer ion. A series of protein standards was chromatographed on a Protein-Pak SP 8 HR (100 × 10 mm) in either 0.02 *M* phosphate (top) or 0.02 *M* acetate (bottom), both at pH 5.5. The same ionic strength gradient from 0 to 0.3 *M* NaCl over 8 column volumes was used with each buffer. Abbreviations as in Fig. 7.

chymotrypsinogen is only sightly affected by this pH change that shifts ribonuclease dramatically. This change in selectivity may reflect the titration of a histidine side chain on the surface of ribonuclease since the change in chromatographic behavior is observed near the p*K* of this amino acid in a protein. Alternatively, since ribonuclease can bind orthophosphate[1], the altered retention may reflect a change in the bound ion. This seems less likely as described below (Fig. 8). The change in selectivity would not have been predicted from the isoelectric points of the proteins. Elution from a cation exchanger should be in order of increasing isoelectric point, and ribonuclease deviates from this rule above pH 5.5[2,3]. It is, in fact, often observed that isoelectric focusing does not accurately predict behavior of a protein sample in ion-exchange chromatography[4–6]. It may, therefore, often be useful to examine a fairly wide pH range, within the limits of protein and column stability, to find the optimum pH for a particular separation. Since such an approach will exceed the useful range of a particular buffer, it is necessary to examine the effect of particular buffer ions on selectivity.

The buffering ion is selected to have a p*K* near the optimum pH for the protein separation. With cation exchangers, phosphate is a suitable buffer from pH 6 to 8, but at lower pH acetate provides better buffering capacity. The effect of these alternative buffer ions was compared at pH 5.5, as shown in Fig. 8. The change in selectivity is most apparent for ribonuclease, but both lysozyme and cytochrome *c* are affected.

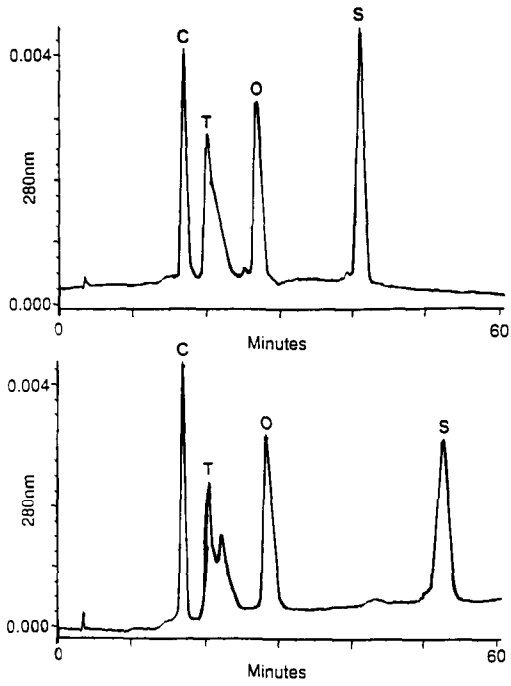

Fig. 9. Selectivity in anion-exchange chromatography as a function of counter ion. A series of protein standards was chromatographed on a Protein-Pak DEAE 8HR (100 x 10 mm) in 0.02 *M* Tris–HCl at pH 7.5. The ionic strength gradient was from 0 to 0.2 *M* sodium chloride (top) or from 0 to 0.2 *M* sodium acetate (bottom) over 8 column volumes. Abbreviations as in Fig. 2.

The increased retention of ribonuclease relative to the other proteins in the mixture may reflect the depletion of orthophosphate that was bound to the protein[1–3], thus exposing cationic amino acid side chains for binding to the packing material.

Since the buffering ion has an effect on selectivity, the counter ion should also profoundly alter the separation. The same ionic strength gradient was compared with chloride and with acetate (Fig. 9). Although conalbumin and ovalbumin are essentially unchanged, resolution of transferrin and its contaminants is improved with acetate. Soybean trypsin inhibitor is eluted much later with acetate, as expected from the elutropic series, but it is surprising that the other standards proteins are not also shifted to longer retention by the same effect.

In addition to the mobile phase components described above, temperature is expected to have an effect on retention. When anion-exchange chromatograms of protein standards at 4 and 25°C are compared (Fig. 10), a change in selectivity is observed. Such alterations are expected but unpredictable since the p*K* of the buffer, the apparent pH, the p*K* of the surface of the ion-exchange resin, the binding constants and the p*K* of each of the ionizable groups of the protein are sensitive to temperature. Commonly, the temperature used for a protein separation is dictated by recovery of biological activity so temperature is not a useful variable for optimizing the separation. However, it may be necessary to readjust other conditions when the chromatographic experiments are shifted between the cold room and the normal laboratory.

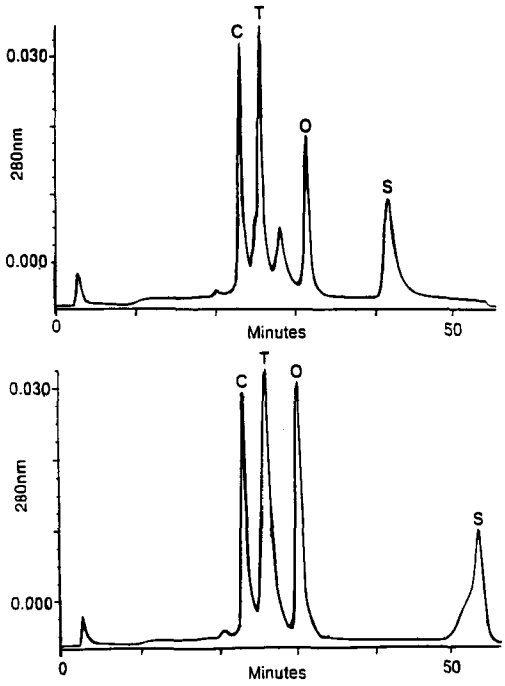

Fig. 10. Selectivity in anion-exchange chromatography as a function of temperature. A series of protein standards was chromatographed on a Protein-Pak DEAE 5PW (75 × 7.5 mm) in 0.02 *M* Tris–HCl at pH 7.5. The same ionic strength gradient from 0 to 0.2 *M* NaCl over 8 column volumes was used at both 4 (bottom) and 25°C (top). Abbreviations as in Fig. 2.

Application of these principles to the development of a particular protein separation involves the preparation of several pairs of buffers. It has proven more convenient and efficient to use the Auto-Blend™ Method[7]. This approach is based on accurate and precise blending of four buffers by the liquid chromatograph. Typically, the A stock is 0.1 *M* acid salt and the B stock is 0.1 *M* basic salt. The relative proportions drawn from the A and B lines determine the pH of the separation. The ionic strength is independently controlled by varying the proportions of the counter-ion stock and the water in the C and D lines, respectively. Both pH and ionic strength are, therefore, determined by entries made in the gradient controller. It is also straightforward to change these parameters independently or simultaneously during the separation.

The Auto-Blend Method was used for the separation of lactate dehydrogenase from crude extracts of mouse liver. As the pH of the buffer was changed, the pattern of protein separation, as judged by the absorbance at 280 nm, also changes. However, the lactate dehydrogenase (LDH) activity was nearly unretained until the pH was raised to 8.6. The retention of LDH relative to the mass of protein in the sample changes dramatically over the narrow range from pH 8.0 to 8.6 (Figs. 11 and 12). Further improvements in the separation can be achieved by reducing the slope of the ionic strength gradient followed by adjustment of the pH in smaller increments. Finally, acetate could replace chloride as the counter ion. These experiments are

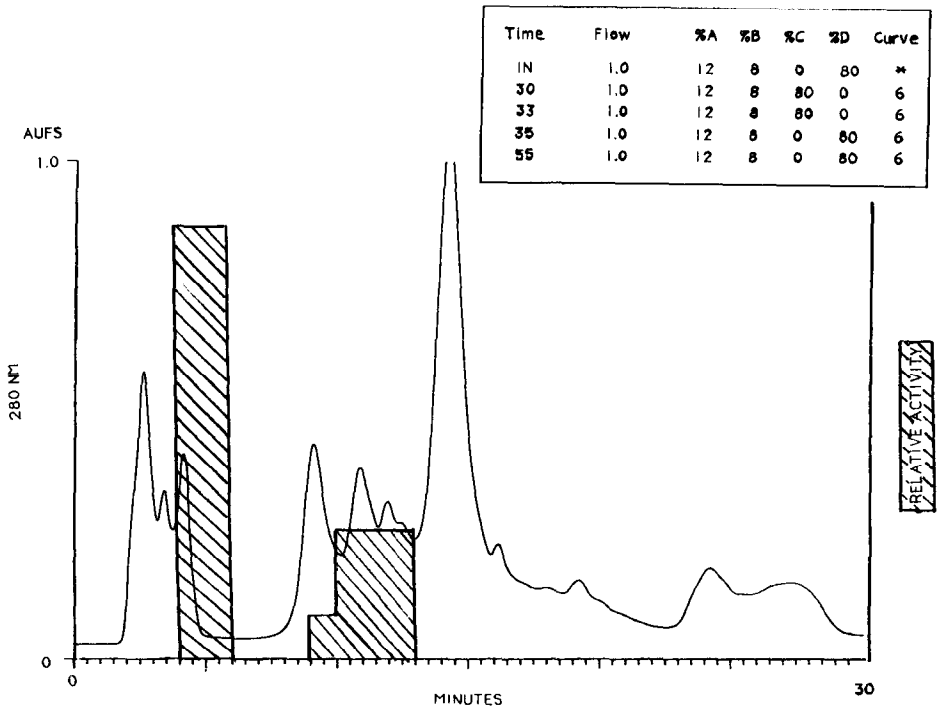

Fig. 11. Anion-exchange chromatography of mouse liver extract (pH 8.02). Conditions as in Experimental section. Enzyme activity (LDH) shown as bars with a diagonal lines and absorbance at 280 nm as continuous tracing.

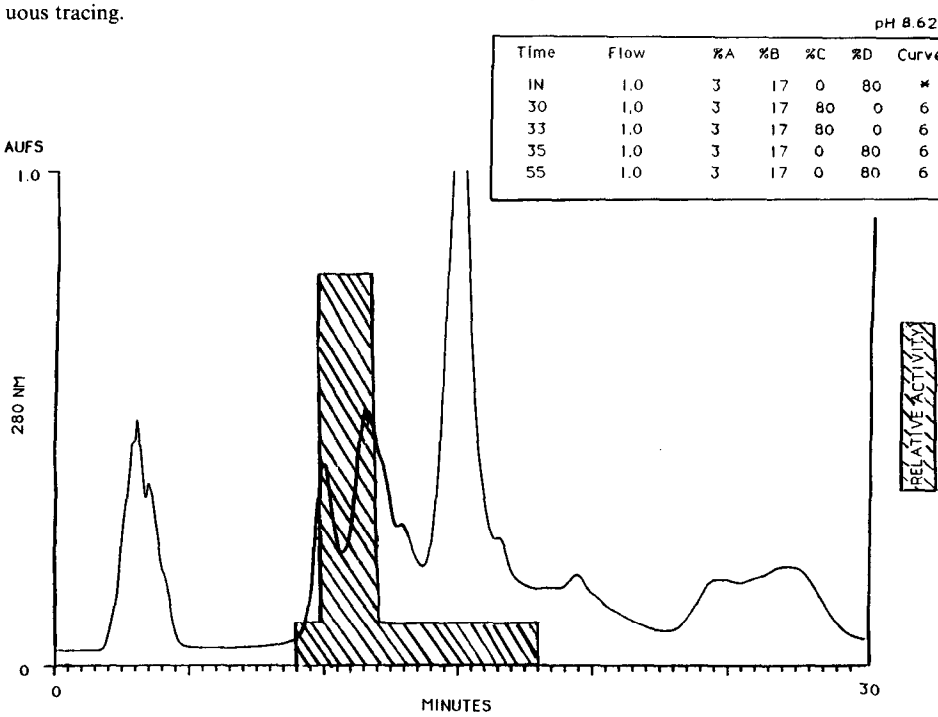

Fig. 12. Anion-exchange chromatography of mouse liver extract (pH 8.62). Conditions as in Experimental section. Enzyme activity (LDH) shown as bars with a diagonal lines and absorbance at 280 nm as continuous tracing.

quickly and conveniently programmed from the keyboard of the gradient controller and can be completely automated by the use of an autosampler.

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