Journal of Chromatography, 294 (1984) 235–246
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,664

EXAMINATION OF KINETIC EFFECTS IN THE HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF GLYCOPROTEINS BY STOPPED-FLOW AND PULSED ELUTION METHODS

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(First received January 10th, 1984; revised manuscript received February 15th, 1984)

SUMMARY

Using a concanavalin A high-performance liquid affinity chromatography system, the effect of slow solute desorption from the silica-bound ligand on protein recovery and peak shape has been investigated. The desorption of glycoproteins from the immobilized concanavalin A was found to be many times slower than the desorption of monovalent carbohydrates. To overcome the kinetic limitations of this chromatographic system, a novel method of protein elution, stopped-flow elution, was developed and used to elute ovalbumin and to examine the kinetic "sluggishness" of the desorption process. The stopped-flow elution method allows the quantitative recovery of purified glycoprotein in a volume of approximately 0.5 ml.

INTRODUCTION

The use of silica-bound biospecific ligands for the chromatographic separation of proteins is a relatively new development in biochemical separations[1]. A review of this method, high-performance liquid affinity chromatography (HPLAC), has recently appeared[2]. Because the peak widths observed in HPLAC greatly exceed, and the plate counts are much less than, those normally encountered in high-performance liquid chromatography (HPLC) under similar conditions of particle size and flow-rate, it seems somewhat premature to refer to this method as a "high performance" technique at this time, but the term has already become well established and will be used here.

Several investigators have attributed the increased peak broadening in HPLAC to slow sample dissociation from the biospecific adsorbant[3,4]. Recently, we have investigated the kinetic characteristics of silica-bound concanavalin A[5]. Concanavalin A, a protein which selectively binds mannose, glucose, and glycoproteins containing these carbohydrate moieties, is a well characterized ligand and has been used extensively in biochemical investigations[6,7]. Using *p*-nitrophenyl-α-D-mannoside and *p*-nitrophenyl-α-D-glucoside as test solutes, we have shown that the rate constant for the dissociation of the immobilized concanavalin A–carbohydrate complex is *ca.* 30

0021-9673/84/$03.00

times smaller than that observed in solution. We have shown that, on 10-μm particles, at a linear velocity of 0.05 cm/sec (1 ml/min), 85% of the observed plate height is due to the slow dissociation of the immobilized concanavalin A–solute complex.

In determining the rate constants for the dissociation of carbohydrates from immobilized concanavalin A, we used the equations of Horváth and Lin[5,8]. This approach allows one to separate the (chemical) kinetic contribution to the total plate height from the non-kinetic contribution and to calculate a rate constant for the chemical process. The approach used is described fully in ref. 5. The relevant equations are given below:

$$H_{\text{kinetic}} = H_{\text{total}} - H_{\text{non-kinetic}} \tag{1}$$

$$H_{\text{kinetic}} = \frac{2k'u_e}{(1 + k_0)(1 + k')^2 k_d} \tag{2}$$

where u_e is the linear interstitial flow velocity, k_0 is the ratio of interparticle void volume to interstitial void volume, k' is the capacity factor for the solute, k_d is the dissociation rate constant (in sec^{-1}) and H refers to the plate height. The total plate heights obtained for simple carbohydrates injected onto the concanavalin A columns were generally in the range of 0.80 to 1.12 mm, corresponding to reduced plate heights ($h = H/d_p$) of 80 to 112. The kinetic contribution to the total plate height for these solutes was 0.68 to 1.00 mm. In contrast, the plate height obtained for a structurally similar, but non-binding, carbohydrate was 0.12 ($h = 12$).

Since the kinetic contribution to plate height is directly proportional to flow-rate, decreasing the flow-rate should produce narrower peaks. This was indeed found to be the case for the elution of monovalent carbohydrates where the rate constant for the dissociation of the immobilized concanavalin A–*p*-nitrophenyl-α-D-mannoside complex is on the order of 0.3 sec^{-1}.

Because glycoproteins are multivalent with respect to available carbohydrate residues, we anticipated that the dissociation of the immobilized concanavalin A–glycoprotein complex would be much slower than the dissociation of the concanavalin A–monosaccharide complex. Eqn. 2 and the results obtained with monovalent solutes can be used to estimate the effect of k_d on the resulting peak width. For example, if k_d for the desorption of a glycoprotein is 10 times smaller than k_d for the desorption of a monovalent carbohydrate, the resulting reduced plate height (at 1 ml/min) would be of the order of 800 to 1120. Alternatively, to achieve a reduced plate height of *ca.* 20 for this solute, a flow-rate of 0.02 ml/min would be required, which is clearly outside the practical range of most HPLC solvent delivery systems.

The above calculation assumes that the desorption of a multivalent glycoprotein from immobilized concanavalin A is an extremely slow process. Although the rate constant for such a process has not been precisely determined, we present chromatographic results in this paper indicating that this desorption is many times slower than the desorption of a simple carbohydrate from immobilized concanavalin A. Because flow-rates less than 0.1 ml/min are required to elute glycoproteins under these conditions, we describe here a novel method of protein elution which allows the protein to desorb under conditions of stopped flow. Note that, according to eqn. 2, a linear flow velocity of 0 will result in no kinetic contribution to band broad-

ening. Using our stopped-flow method, we have investigated the elution of ovalbumin from a concanavalin A column and determined conditions which allow this glycoprotein to be eluted as a single narrow peak. The stopped-flow method is a general method of protein elution and should be useful in all forms of HPLAC.

The methods described here involve the use of the "minicolumns" recently described by Walters[9,11]. The "minicolumn" (0.5 × 0.46 cm I.D.) used in these studies requires only 35 mg of packing material and thus minimizes the cost of using expensive affinity ligands. In addition, the back pressure generated by this column was found to be only 30 p.s.i. At such low pressures, the use of a relatively inexpensive peristaltic pump becomes an attractive alternative.

EXPERIMENTAL

Materials

Concanavalin A (Con A, Type IV), α-methyl-D-mannoside (Grade III, αMDM), ovalbumin (OVA, Grade VI), and horseradish peroxidase (HRP, Type VI) were obtained from Sigma (St. Louis, MO, U.S.A.). Prior to immobilization, the Con A was purified by the method of Cunningham *et al.*[10] to isolate the intact subunits. LiChrospher 500 (Batch No. YE 496, pore diameter 500 Å, particle diameter 10 μm) was obtained from E. Merck (Darmstadt, F.R.G.). γ-Amino-propyltrimethoxy silane was obtained from Silar (Scotia, NY, U.S.A.). All other chemicals were reagent grade.

Preparation of biospecific adsorbant

Con A was immobilized on LiChrospher 500 by the method previously described[5]. The amount of Con A bound to the silica was determined by the difference in absorbance of the Con A solution at 280 nm before and after immobilization. The amount of Con A detected in the washings of the silica was also included in this determination. The amount of Con A bound to the silica (in milligrams of Con A per gram of silica) was found to be 72 mg/g.

Column packing and chromatographic conditions

The immobilized Con A was manually slurry-packed into 0.5 cm × 0.46 cm I.D. "minicolumns". The estimated total liquid volume in the column was 0.07 ml. An HPLC system comprised of an IBM LC/9533 solvent delivery system, an injector (Rheodyne 7126) fitted with a 20-μl loop, two Rheodyne column switching valves (7040 and 7010), a UV–visible spectrophotometer (Hitachi) equipped with a 10-μl flow cell (Altex) and a Hewlett-Packard 3390A integrator were used. The switching valves were included to provide the capability of forward or reverse flow in the column and to direct the eluent flow to a column bypass path. The configuration of this system is shown in Fig. 1. The detection wavelength used was 240 nm for both OVA (ovalbumin) and HRP (horseradish peroxidase). At 240 nm, there is some baseline interference from the absorbance of the αMDM in the mobile phase. The measured absorbtivity for αMDM at this wavelength is 0.04 cm^{-1} M^{-1}. The mobile phase used was 0.02 *M* sodium phosphate, pH 6.0, containing 0.5 *M* sodium chloride, 0.01 *M* magnesium chloride, and 0.001 *M* calcium chloride, proposed stabilizers of Con A. The competing mobile phase consisted of 0.1 *M* αMDM dissolved in the above mobile phase. The flow-rate was 0.5 ml/min, except where noted.

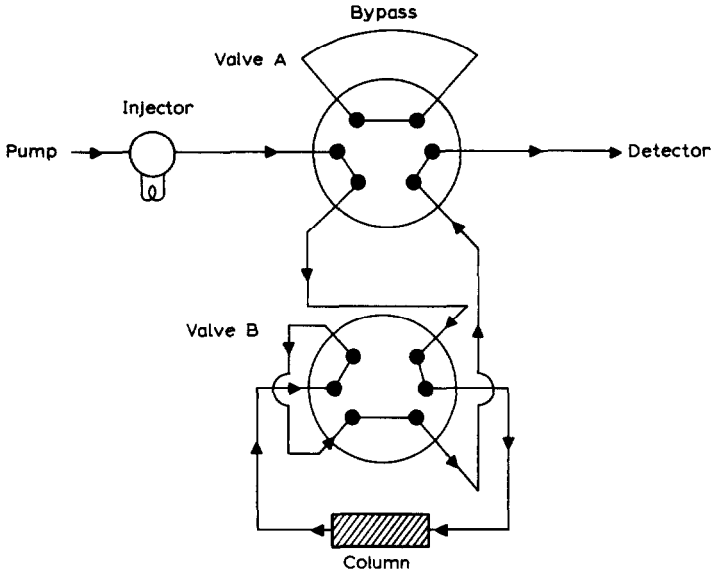


Fig. 1. Configuration of system (in forward flow/column mode). Valve A (Rheodyne 7010) is used to select bypass or column mode. Valve B (Rheodyne 7040) is used to select forward or reverse flow in the column.

Elution procedures

Two different methods of protein elution, pulsed and stopped flow, were investigated. In both cases, the protein sample was injected onto the column in the non-competing mobile phase and the non-binding fraction was first eluted.

Pulse elution. An interval of 2 min (or 4 min where noted) after sample injection, 20 μl of strong mobile phase (0.1 *M* αMDM) were injected onto the column. These pulses were then injected every 2 (or 4 as noted) min until the height of the resultant peak was no longer different from a series of blank injections that preceded the protein injection.

Stopped-flow elution. (See Fig. 2). In this procedure, a sample is injected (point A) onto the column and the non-binding protein is eluted with weak (non-competing) eluent for a period of precisely 2 min. At the end of this time (point B), the flow of eluent is diverted around the column via the bypass loop using valve A (see Fig. 1). At the same time, (point B), the eluent is changed to the strong mobile phase (0.1 *M* αMDM). The flow to the detector must be maintained at all times in order to prevent gross discontinuities in the baseline. Once the new baseline is fully established (point C) indicating a steady state concentration of αMDM in the eluent, flow is redirected to the column for precisely 10 sec. Note the appearance of a small perturbation in the baseline due to the momentary interruption of flow when the valve is actuated (point D). At the end of the 10 sec, flow is again diverted through the bypass loop and the column is not flushed. In preliminary experiments, 10 sec were found to be sufficient to fill the column with competing mobile phase at a flow-rate of 0.5 ml/min. The strong mobile phase then causes the desorption of the bound protein from Con A by competition for binding sites on the immobilized lectin. Because there is no flow through the column while sample desorption is taking place, there can be ab-

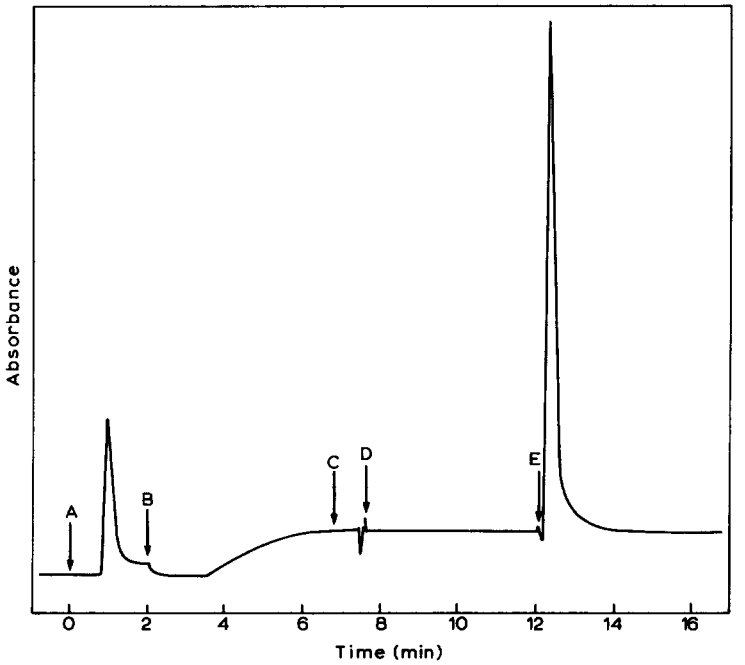


Fig. 2. Stopped-flow elution procedure. Column: 0.5 × 0.46 cm I.D., 72 mg of Con A per gram of silica, 10 μm, nominal pore size 500 Å. Mobile phase: pH 6.0, 0.02 *M* sodium phosphate, 0.5 *M* sodium chloride, 0.01 *M* magnesium chloride, 0.001 *M* calcium chloride. Flow-rate, 0.5 ml/min; sample, 23 μg OVA. See text for explanation.

solutely no kinetic contribution to band broadening[8,12,13] despite the sluggishness of the desorption process. The time period during which desorption is allowed to occur is designated t_s (static time). At the end of period t_s (point E), the flow of strong mobile phase is re-routed to the column and the desorbed protein is eluted. In some experiments, the flow was introduced in the "backflush" mode (see below) at point E using valve B.

Determination of protein recovery

The amount of total protein sample recovered under a given set of stopped-flow conditions was determined by comparing the area of the peak obtained after elution through the affinity column (A_{col}) with the area of the peak obtained after elution through an open tube (A_{ot}). Since the commercial preparations of OVA and HRP were impure, the area of the non-binding peak eluted initially in the non-competing mobile phase (A_{nb}) was subtracted from the total open tube area to obtain the open tube area that could be attributed to the binding protein. The per cent of binding protein eluted under a given set of conditions was then calculated as follows:

$$\text{Per cent eluted} = \frac{A_{col}}{(A_{ot} - A_{nb})} \cdot 100\% \tag{3}$$

The calculation of fractional recovery under pulsed elution conditions was the same

as that shown above with the exception that the area of the αMDM in the pulse itself was subtracted from A_{col}.

RESULTS AND DISCUSSION

Prior to investigating the elution of glycoproteins from silica-bound Con A, the elution of multivalent carbohydrates was investigated. Although these results are not shown here, the peak width obtained for bivalent mannobiose was much larger than that obtained for the monovalent carbohydrates at the same flow-rate. The peak obtained for mannotriose was barely distinguishable from baseline (essentially infinite peak width) even at flow-rates as low as 0.2 ml/min. These results suggested that the desorption of multivalent solutes was many times slower than the desorption of monovalent solutes and that simply decreasing the flow-rate was not sufficient to produce reasonable peak widths.

Accordingly, conventional HPLC techniques such as isocratic or gradient elution cannot be used for the HPLAC analysis of OVA or HRP. Fig. 3 shows the effect of applying a step gradient of 0 to 0.1 *M* αMDM ten minutes after injection of an OVA sample. The non-binding components of the OVA sample are immediately eluted in the non-competing mobile phase, but only a small fraction of the bound OVA is eluted upon application of the gradient. The extremely slow return to baseline in Fig. 3 indicates the magnitude of the sluggishness of the kinetics observed in this system. When a linear gradient (0 to 0.1 *M* αMDM) is run over a period of 30 min, the bound OVA peak is barely distinguishable from the baseline. Injection of either

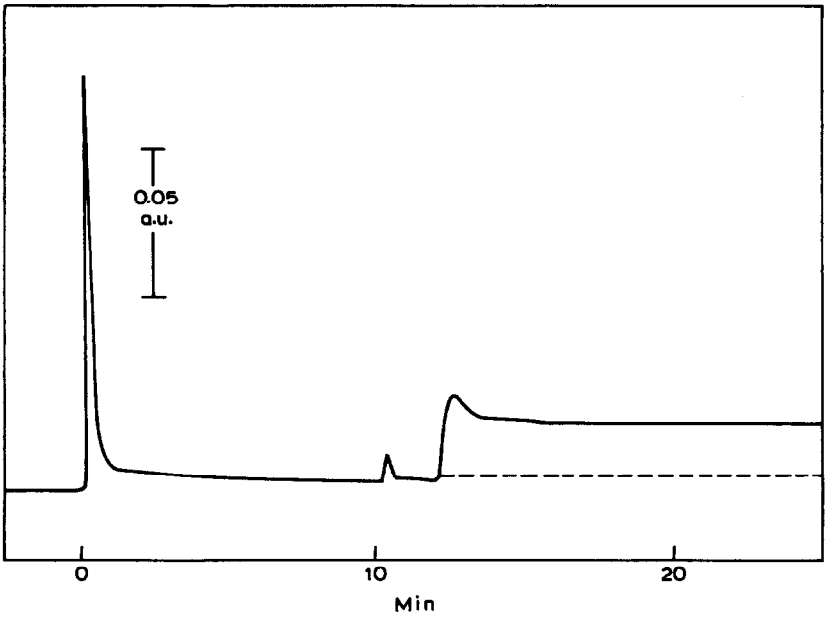
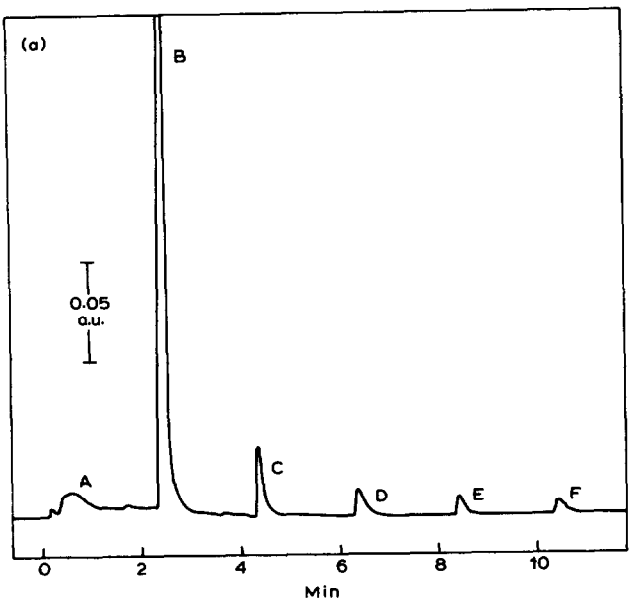


Fig. 3. Step gradient elution of OVA. Column and mobile phase as in Fig. 2. Flow-rate, 0.5 ml/min; sample, 20 μl of 25 μ*M* OVA (23 μg protein injected). An interval of 10 min after sample injection, mobile phase changed to 0.1 *M* αMDM in above mobile phase. (- - - -) Baseline due to αMDM in mobile phase.



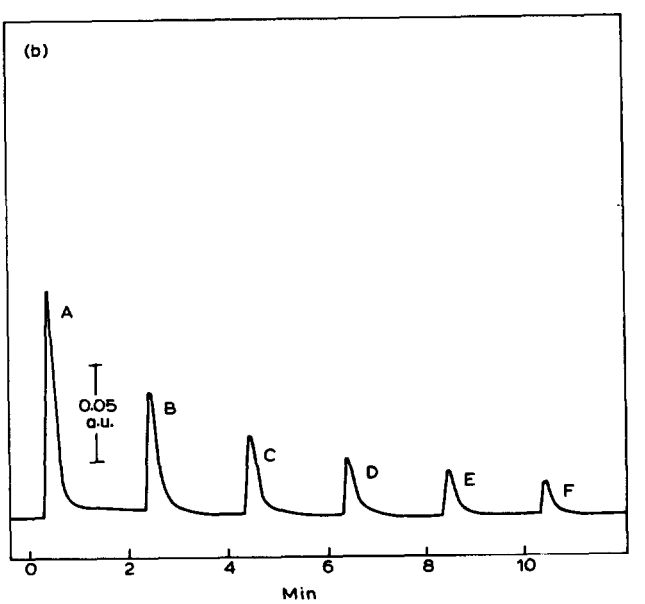


Fig. 4. (a) Pulsed elution chromatogram of HRP. Column and mobile phase as in Fig. 2. Flow-rate, 0.5 ml/min; sample, 20 μl of 25 μM protein injected. Injection of 20 μl of 0.1 M αMDM 2 min after sample injection and every 2 min thereafter. Peak A: unretained protein; peaks B, C, D, E, F: retained protein. (b) Pulsed elution of OVA. Same conditions as in (a).

HRP or OVA onto a Con A column pre-equilibrated with the competing mobile phase results in the appearance of a single unretained peak. In the case of HRP, this peak represents 99% of the injected protein and 98% in the case of OVA. This result indicates that the injected protein is incapable of displacing the competing sugar bound to the Con A sites on the column within the time that the protein pulse passes through the column (*ca.* 8 sec at 0.5 ml/min).

Pulse elution of HRP and OVA

The results of applying the pulse elution technique to samples of HRP and OVA are shown in Fig. 4. Although 93% of the HRP can be eluted with a single pulse, even after three pulses, less than 60% of the OVA has been eluted. To exclude the possibility that we were fractionating different forms of OVA by this method, we investigated the effect of flow-rate on the amount of OVA obtained with each pulse. These results are shown in Table I. The drastic effect of flow-rate on the amount of OVA eluted with each pulse indicates that the observed elution pattern is kinetic in nature, and not a thermodynamic separation of different forms of OVA. At the lower flow-rates, the residence time of the competing pulse in the column is longer, and thus more protein can be eluted. Even at 0.2 ml/min (where the residence time of the pulse in the column is 21 sec), only 25.4% of the bound OVA can be eluted with a single pulse of sugar.

Stopped-flow elution procedure for OVA

The results of the pulsed elution experiments indicated that, to achieve quantitative protein recovery of the bound OVA, flow-rates less than 0.2 ml/min (*i.e.*, pulse residence times longer than 21 sec) would be necessary. Since the required flow-rate is too low for reproducible flow with most conventional solvent delivery systems, we chose to stop the flow entirely rather than simply decrease it.

The stopped-flow elution technique described above was used to investigate the rate of OVA debinding from the immobilized Con A in the presence of 0.1 *M* αMDM. The "static time", t_s, is the time allowed for the Con A–OVA complex to

TABLE I

EFFECT OF FLOW-RATE ON PROTEIN RECOVERY (%) IN THE PULSED ELUTION OF OVALBUMIN

Column: 0.5 × 0.46 cm I.D., 72 mg of Con A per gram of silica, 10 μm, nominal pore size 500 Å. Mobile phase: pH 6.0, 0.02 *M* sodium phosphate, 0.5 *M* sodium chloride, 0.01 *M* magnesium chloride, 0.001 *M* calcium chloride. Flow-rate, 0.5 ml/min; solute, 20 μl of 25 μ*M* OVA (23 μg injected).

*Pulse number**	*Flow-rate (ml/min)*		
	0.2	*0.5*	*2.0*
1	25.4**	15.8	7.9
2	18.6	14.1	5.5
3	15.1	8.3	4.3

* 20-μl pulses of 0.1 *M* αMDM injected at 4-min intervals.

** Per cent eluted $= \frac{A_{col} - A_{MDM}}{A_{ot} - A_{nb}} \cdot 100.$

dissociate under static conditions in the presence of 0.1 *M* αMDM. Fig. 5 illustrates the differences in peak shape obtained as t_s is varied. As the value of t_s decreases, the total peak area is diminished and the peak tailing increases dramatically. The peak widths at half height and the peak asymmetry factors (measured at 10% of peak height[14]) are given in Table II. The peak tailing observed at short reaction times is characteristic of a slow mass transfer process[15]. In Fig. 6, the amount of OVA recovered (calculated from eqn. 3) as a function of t_s is shown. Because the curve appears to reach a plateau at a value of t_s equal to 8 min, at least 8 min are required for the column to reach an equilibrium distribution under these conditions. The amount of protein recovered for "static" times less than 20 sec is not shown because the irregularity of the peak shapes at these times drastically reduced the accuracy of the integrated areas. For stopped-flow times greater than or equal to 8 min, the amount of OVA recovered as calculated from eqn. 1 is 78%. Because the area of these very asymmetric peaks, as reported by the integrator, does not include a large fraction of the peak "tail", a second data acquisition system was used to digitally collect the chromatographic data. Using this system the baseline could be more accurately determined and the peak tail included in the area determination. By this method the long-time recovery of OVA was determined to be 96% (± 2%).

Because the OVA sample binds so strongly to the immobilized Con A, and because the amount of Con A in the column vastly exceeds the amount of sample protein, it is likely that the protein binds only in a small zone at the head of the chromatographic column. In this case, the OVA could then possibly readsorb onto vacant Con A binding sites as it moved down the column. This could contribute to

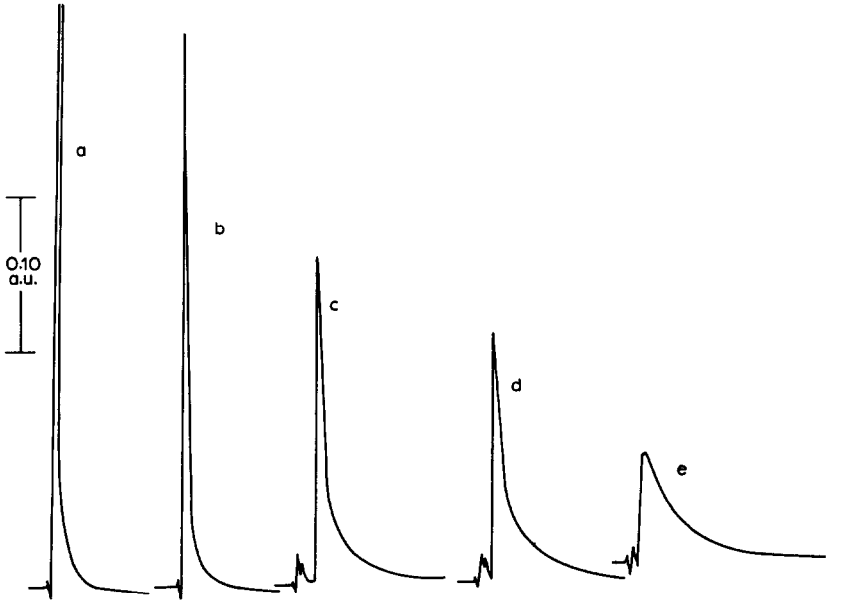


Fig. 5. Stopped-flow elution of OVA: effect of t_s on peak shape. Column and mobile phase as in Fig. 2. Flow-rate, 0.5 ml/min; sample: 20 μl of 25 μ*M* OVA (23 μg protein injected). Peak a: t_s = 8 min, peak b: t_s = 2 min, peak c: t_s = 20 sec, peak d: t_s = 10 sec, peak e: t_s = 0.

TABLE II

EFFECT OF REACTION TIME, t_s, ON PEAK WIDTH AND ASYMMETRY FACTOR IN THE STOPPED-FLOW ELUTION OF OVALBUMIN

Column: 0.5 × 0.46 cm I.D., 72 mg of Con A per gram of silica, 10 μm, nominal pore size 500 Å. Mobile phase: pH 6.0, 0.02 *M* sodium phosphate, 0.5 *M* sodium chloride, 0.01 *M* magnesium chloride, 0.001 *M* calcium chloride. Flow-rate, 0.5 ml/min; solute, 20 μl of 25 μ*M* OVA (23 μg injected).

t_s (sec)	$W_{1/2}$ (ml)	a/b
0	0.39	17.0
10	0.12	14.5
20	0.087	11.5
40	0.083	4.0
60	0.083	2.3
120	0.039	1.5
240	0.031	1.6

the observed peak asymmetry. To test this hypothesis, we performed the stopped-flow experiments described above in a "backflush" mode. The OVA sample was injected onto the head of the column, the column was filled with competing mobile phase in the forward flow mode, but after completion of the "static" time, the mobile phase flow was directed to the rear of the column and flow was then continued in the reverse direction. Since the OVA sample must exit the column from the same end

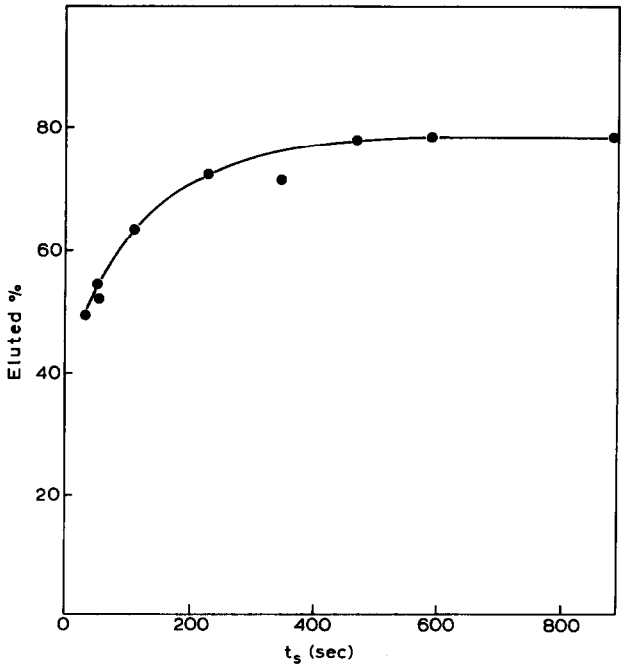


Fig. 6. Effect of reaction time, t_s, on OVA recovery: stopped-flow elution. Column and all conditions as in Fig. 4. Per cent eluted calculated using eqn. 3.

that it entered with this flow pattern, there is essentially no possibility for OVA readsorption prior to elution. Both forward flow and reverse flow experiments were done for reaction times of 5, 10, and 15 min. The amount of OVA recovered and the peak shape were essentially the same for both the forward and reverse flow modes. This finding supports our earlier observation that OVA is incapable of displacing the bound αMDM within the time scale of our experiments. Under different conditions, however, (such as less competing sugar in the mobile phase or a different sample protein), the backflush method is a convenient technique to prevent sample readsorption.

CONCLUSIONS

In this work, the elution of OVA from an immobilized Con A HPLAC minicolumn was shown to be kinetically, rather than thermodynamically determined. The kinetics of the OVA desorption from immobilized Con A were found to be extremely slow with respect to a chromatographic time frame. A "stopped flow" method of protein elution was developed to overcome the slow dissociation kinetics of the Con A–OVA complex. Using this procedure, purified OVA can be eluted in a volume of 0.5 ml (baseline to baseline) and with a total separation time of *ca.* 15 min. The eluted glycoprotein is present at a much higher concentration by this method than it is by a conventional continuous flow procedure. This aspect of the method could be very important in a practical sense, where the isolation of all mannose and glucose rich glycoproteins in a biological sample by this technique could be an early step in a total protein purification.

Variation of the eluent strength in conjunction with the stopped-flow reaction time could be used to elute glycoproteins other than OVA from these Con A columns. The stopped-flow method seems to be a promising method of protein elution in HPLAC, or in other chromatographic systems, where the attainment of equilibrium is a slow process.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge R. R. Walters for many helpful discussions concerning this work. Financial support for A. J. Muller was provided by the National Science Foundation (CHE 8217363).

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