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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF PROTEINS WITH STRONG ACIDS

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SUMMARY

Small organic acids are generally used as pairing agents at less than 1% concentration in reversed-phase chromatography (RPC) of proteins. When the protein is very hydrophobic and insoluble, as in the case of membrane proteins, up to 60% aq. formic acid has been used. This paper reports a study of the influence of acid concentration on both chromatographic retention and protein structure in RPC.

Chromatographic retention increases in proportion to the concentration of organic acid in the mobile phase up to some intermediate concentration. Use of still higher concentrations of acid results in a sharp drop in chromatographic retention with a change in selectivity. The data indicate that the structure of proteins in strong acids are different from that in weak acids. This work examines the reason for this decrease in chromatographic retention at formic and trifluoroacetic acid concentrations above 30% (v/v). Spectroscopic studies show that protein conformation continuously changes with the addition of acid.

INTRODUCTION

Retention and elution of proteins in reversed-phase chromatography (RPC) involves conformational changes of the solute and generally denaturation. The nature of the solvents used in this chromatographic mode, and the interaction of the polypeptides with the sorbent are the main causes for this denaturation[1]. The most popular solvents for RPC consist of a very acidic aqueous solution and a gradient of organic solvent, particularly when silica-based materials are employed as the sorbent. Protonation of the residual silanol groups on the support diminishes ionic interactions with the basic amino acid residues of the proteins. Moreover, small organic acids form ion pairs with these basic sites. Recently, a large-pore poly(styrene–divinylbenzene) material has become commercially available that can be used for RPC of biological compounds[2,3]. The chemical stability of this material is an advantage in work with strong acids and bases[4]. Moreover, this new packing does not contain silanol groups. It should be noted, however, that the use of trifluoroacetic acid (TFA) at concentrations of 0.1 to 1% with acetonitrile as modifying solvent often gives the best chromatographic results, even with organic resins which contain no silanol groups[5].

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The process by which proteins are denatured in RPC has been a subject of intense investigation in the past few years. The response to the denaturants both in solution and on the column varies with each protein[6,7]. As determined by coupling RPC with other chromatographic modes, even the rate of unfolding and the extent of denaturation following the removal of acids and organic solvents is protein-specific[8,9]. Depending on the degree of unfolding and the reversibility of the process, proteins may be eluted with multiple or broad peaks, as has been shown in several studies[10–12].

All of the work cited above has been done with dilute acids (0.1–1%). However, these conditions are of limited utility in the chromatography of large, hydrophobic proteins. Cell membranes or viral envelopes are not soluble under such conditions, and cannot be eluted[13]. It is necessary to work at very high acid concentrations to solubilize the proteins in these cases. Formic acid at concentrations ranging up to 60% has been found of much greater utility than the more hydrophobic TFA normally used[14,15]. To date, little work has been done to understand why such conditions are more successful for the chromatography of these proteins. Obviously, such severe conditions must have a profound effect on protein structure. This work examines the influence of high concentrations of acid on protein chromatography. Maximum retention was obtained for all the proteins studied at intermediate acid concentrations. Solvent effects or conformational changes could induce such a phenomenon. Spectroscopic methods were employed to determine whether conformational changes of proteins occur in such media. This paper will show that the conformation of proteins changes with the concentration of acid in the mobile phase. The balance between hydrophobic and hydrophilic amino acid residues at the surface of the protein seems the main cause for the retention changes of the polypeptides in RPC.

MATERIALS AND METHODS

Equipment

The chromatographic system consisted of a Varian 5500 gradient pumping system (Varian, Walnut Creek, CA, U.S.A.), a Valco (Houston, TX, U.S.A.) Model C6U injector with a 2-, 40- or 100-μl sample loop and an LC 85B variable-wavelength UV detector (Perkin Elmer, Norwalk, CT, U.S.A.) operated at 280 nm. A Hewlett-Packard (HP) (Palo Alto, CA, U.S.A.) Model 1040A photodiode-array detector was used for the determination of absorbance ratios and second-derivative spectroscopy. This detector was equipped with an HP85B personal computer, an HP 9121 P/S disc drive, and an HP 7470A graphics plotter. Second derivatives were processed with the HP Data Evaluation II software. The flow-rate was 1 ml/min unless otherwise specified.

RPC was performed on poly(styrene–divinylbenzene) PLRP-S, 8 μm, 300 Å (1000 Å for IgG) (Polymer Labs., Shropshire, U.K.). Columns were packed in 2-propanol, at 3000 p.s.i. with a Shandon column packer (Sewickley, PA, U.S.A.). Dimensions of the columns were 5 × 0.46 cm I.D. Elution was achieved with linear gradients of 40 min for all the retention measurements and 20 min for the spectroscopic measurements. Except for Fig. 1, eluent B was pure acetonitrile in all RPC experiments. The amount of protein injected varied between 10 and 20 μg for all proteins, except IgG, where 250 μg were injected. Each retention value is the result of at least two reproducible values.

Cation-exchange chromatography was performed on a SynChropak CM 300 column, 15 × 0.41 cm I.D., from SynChrom (Lafayette, IN, U.S.A.).

Reagents

HPLC-grade acetonitrile came from American Burdick & Jackson (Muskegon, MI, U.S.A.). TFA (99%) was from Aldrich (Milwaukee, WI, U.S.A.). Formic acid 88%, from Fischer Scientific (Fair Lawn, NJ, U.S.A.) was used for all the experiments, except for the determination of absorbance ratios and second-derivative spectroscopy, where 99% formic acid from Aldrich was employed. Proteins and peptides were purchased from Sigma (St. Louis, MO, U.S.A.). Acid solutions were filtered through a Whatman GF/C (1.2 μm) filter (Hilboro, OR, U.S.A.).

Buffers for cation exchange were prepared with mono- and dibasic potassium phosphate (Mallinckrodt, Paris, KY, U.S.A.) and sodium chloride (Fischer Scientific). Solutions were filtered through a Rainin Nylon-66 (0.45 μm) filter (Woburn, MA, U.S.A.) and degassed. Recovery of IgG was determined with the Bio-Rad (Richmond, CA, U.S.A.) protein assay reagent.

Ribonuclease A and IgG recoveries

Protein fractions were collected after RPC. Solutions containing 60% and 80% aq. formic acid were dialyzed overnight against 200 and 400 volumes of water, respectively. Samples were then dialyzed twice for 5 h against 200 volumes of 10 m*M* phosphate buffer. The fraction collected with 0.1% acid was dialyzed directly against phosphate buffer, as described above. All dialysates were then lyophilized and resolubilized in water. Protein concentrations were determined by the Bio-Rad micro assay. The 100% control was prepared by disconnecting the column and collecting the protein sample directly after the injection valve.

Refolding of ribonuclease A and ovalbumin

The procedure used for the collection, dialysis, and lyophilization was identical to that for IgG. Samples were chromatographed on an ion-exchange column with a 30-min gradient from 10 m*M* phosphate buffer (pH 7) to 1 *M* sodium chloride in 100 m*M* phosphate buffer (pH 7). Ribonuclease A activity was measured by the method recommended by the enzyme supplier with the exception that the solutions used were fifteen times more concentrated than specified by the supplier.

RESULTS AND DISCUSSION

Chromatographic results

Fig. 1 reports the retention of various proteins as a function of formic acid concentration. The retention of all proteins examined gradually increased to a maximum and then began to decline slowly, with the exception of ovalbumin (OVA). The longer a protein was retained, the higher the concentration of acid required before its retention began to decrease. The normal practice in RPC of polypeptides is to add acid to both the aqueous and organic solvent. When the concentration of acid is low (< 1%), the addition of acid to the organic eluent does not significantly change its concentration. In contrast, addition of 30–80% aq. formic acid to the organic solvent drastically changes it. This make it difficult to interpret the results at high concentra-

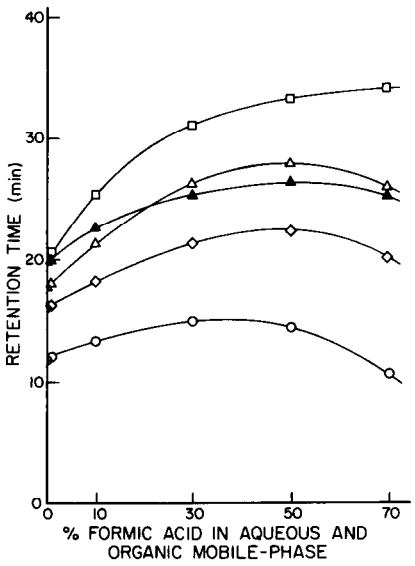

Fig. 1. Retention of various proteins and heme *vs.* the percentage of formic acid in the aqueous and organic mobile phases. For chromatographic conditions see Materials and Methods section. □ = Ovalbumin; ▲ = heme; △ = apomyoglobin; ◇ = lysozyme; ○ = ribonuclease A.

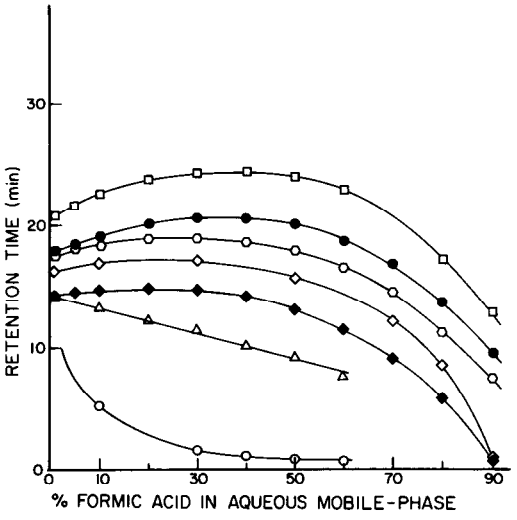

Fig. 2. Retention of various proteins and peptides *vs.* the percentage of formic acid in the aqueous mobile phase. For chromatographic conditions see Materials and Methods section. □ = Ovalbumin; ● = α-chymotrypsinogen-A; ⬡ = soybean trypsin inhibitor; ◇ = lysozyme; ◆ = cytochrome *c*; △ = hippuryl-L-phenylalanine; ○ = N-acetyl-L-tyrosine.

tions of acid. Therefore, the same experiment was repeated without acid in the organic mobile phase (Fig. 2).

When no acid was added to the organic mobile phase, maximum in retention of all proteins was achieved with *ca.* 30% aq. formic acid in the mobile phase. Maxima of these curves, as determined by the first derivative, are given in Table I and reveal that the position of the maximum is related to the retention magnitude (and size) of the protein.

The retentions of two small peptides, hippuryl-phenylalanine and N-acetyl-L-tyrosine, are also shown in Fig. 2. The retentions are maximal at low concentrations of formic acid and decrease with increasing amounts of acid. This is probably because the solvophilic power of formic acid is slightly higher than that of water. Therefore, the increasing retention observed for the proteins studied cannot be explained easily by this solvent phenomenon.

In Fig. 3 the lysozyme retention is plotted against the acid concentration when formic acid is substituted by TFA. TFA is a well-known pairing agent, used in RPC[16] and in size-exclusion chromatography (SEC), where retention is related to the size and charge of the proteins[17]. These curves reveal two phenomena: (i) the curve shape is similar for these two acids and (ii) the increase in retention is larger with TFA than with formic acid. The similarity in curve shapes indicates that the phenomenon observed is not specific for formic acid. One explanation for the second observation may be that the hydrophobicity of TFA is higher than that of formic acid. The more hydrophobic the ion-pairing agent, the higher the retention in RPC[18,19].

The ion pairs formed by acids with the protonated amine groups of the proteins could explain the initial retention increase. The hydrophobic part of the ion-paired acid would increase the surface contact of the solute with the stationary phase and, concomitantly, the retention. Another explanation could be based on a denaturation effect. The more acid is added, the more the proteins are denatured. The hydrophobic interior of the proteins would become more accessible to the stationary phase at high concentrations of acid and this increases chromatographic retention. Retention would continue to increase with the addition of acid until the protein has reached some maximum state of acid-induced unfolding. The decrease in retention at still higher concentrations of acid could be explained by the greater solvophilic power of formic acid relative to water.

To further confirm the ion-pairing and protein unfolding roles of these acids at

TABLE I

PERCENT ACID AT RETENTION MAXIMUM FOR VARIOUS PROTEINS

For chromatographic conditions see Fig. 2.

Proteins	*MW*	*Formic acid (%)*
Ovalbumin	45 000	38 ± 2
α-Chymotrypsinogen A	25 000	35 ± 2
Soybean trypsin inhibitor	20 100	27 ± 2
Apomyoglobin	17 500	28 ± 2
Lysozyme	14 300	26 ± 2
Cytochrome *c*	12 200	22 ± 2

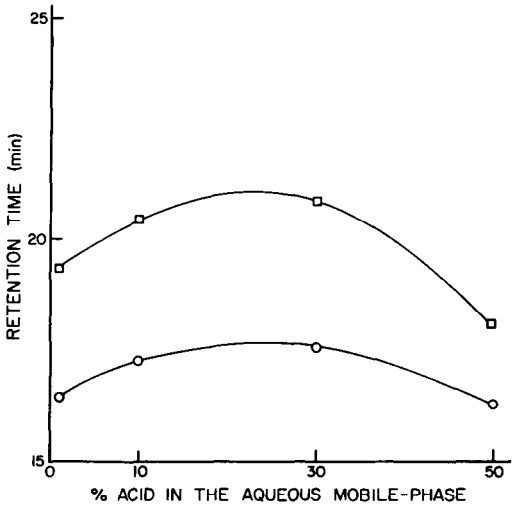

Fig. 3. Retention of lysozyme *vs.* the percentage of acid in the aqueous mobile phase. For chromatographic conditions see Materials and Methods section. □ = TFA; ○ = formic acid.

high concentration, we studied the influence of acid on the retention of a solute possessing a rigid three-dimensional structure in strong acid. It is well known that under acidic conditions, in the presence of organic solvent, myoglobin loses its heme[20]. Consequently, this protein gives two peaks in RPC, one corresponding to the apoprotein, the other corresponding to the free heme (solute possessing a rigid three-dimensional structure). In Fig. 4, the retention of these two solutes are plotted against the concentration of formic acid. Two different retention curves are obtained. While the curve obtained for apomyoglobin is similar to the one obtained for the proteins

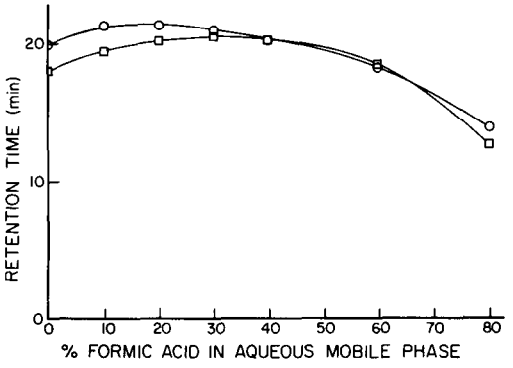

Fig. 4. Retention of apomyoglobin and its heme *vs.* the percentage of formic acid in the aqueous mobile phase. For chromatographic conditions see Materials and Methods section. □ = Apomyoglobin; ○ = heme.

previously studied, it differs for the heme. Its retention first increases with acid concentrations, this is probably due to its ability to bind acid ligands[21], but the maximum retention is reached at a lower acid concentration (10%) than for the proteins. Moreover, the shape of the curve after the maximum is somewhat different from that observed for the proteins in Fig. 2. The same curve shape and maximum retention were obtained with polylysine, a polypeptide capable of forming a large number of ion pairs (data not shown).

These results show that, even if the ion-pairing effect increases when acid is added to the mobile phase, this does not explain the retention of proteins when the concentration of acid is higher than 10%. It is probable that protein structure plays a role in the increase in retention observed at acid concentrations higher than 10%.

Spectroscopic studies

Absorbance ratio measurements. When conformational changes occur in a protein, different amino acid residues become more or less exposed to the solvent. If the aromatic residues are affected by these changes, the UV absorbance of the polypeptide will vary. Consequently, these variations can be monitored by observing the shifts induced in the UV spectrum of the protein. Each of the three aromatic amino acid residues in proteins has a different UV spectrum[22]. If two wavelengths are selected at the absorbance maximum of the aromatic amino acid residues, a ratio of the two will indicate which amino acids become exposed to the surface and which are inside the protein. This technique has previously been applied successfully in following the unfolding of β-lactoglobulin in hydrophobic-interaction chromatography[23]. That work[22,23] has shown that the contributions of the three aromatic amino acid residues to the UV spectra were: 292 nm, tryptophan only; 274 nm, tryptophan and tyrosine; 254 nm, tryptophan, tyrosine, and phenylalanine.

The ratios of these wavelengths have been calculated for various proteins, at different concentrations of acid. The two proteins used in this study contain different relative amounts of the aromatic amino acid residues (Table II). Ribonuclease A was chosen because it lacks tryptophan. The strong absorbance of this aromatic amino acid usually prevents the observation of any changes for phenylalanine. Consequently, this protein allowed studies of changes in the environment of phenylalanine. Lysozyme has a ratio Trp/Tyr of 2, which allows studies of changes in the exposure of these two aromatic amino acid residues to the surface, as seen in Fig. 5. A reorientation of aromatic amino acids seems to occur at an acid concentration around 30%, as seen in all the curves.

TABLE II

AROMATIC AMINO ACID RESIDUES IN VARIOUS PROTEINS

Protein	*Number of residues*		
	Phe	*Tyr*	*Trp*
Ribonuclease A[27]	3	6	0
Lysozyme[27]	3	3	6
Ovalbumin[26]	20	9	3

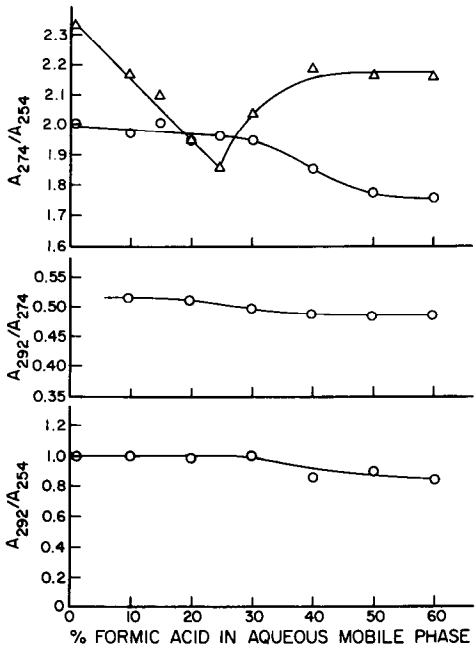

Fig. 5. Influence of the amount of formic acid on the spectroscopic properties of various proteins. For chromatographic conditions see Materials and Methods section. △ = Ribonuclease A; ○ = lysozyme.

With ribonuclease A, the A_{274}/A_{254} ratio decreased when the initial amount of formic acid was added. This suggests a change in conformation, resulting in phenylalanine residues becoming more exposed at the surface of the protein. This is expected in denaturation. At formic acid concentrations higher than 25%, the ratio suddenly increased and remained stable. This suggests either an increase of the absorbance at 274 nm or a decrease at 254 nm. Either exposure of tyrosine residues to the surface or movement of the phenylalanine residues back into the interior of the protein, or both, could result in such a spectral change. The data do not allow us to decide which of these phenomena are occurring.

Lysozyme contains all three aromatic amino acid residues, interpretation of the results obtained with this protein is consequently more complex. However, a drop in the A_{274}/A_{254} ratio was noticeable when the level of acid was higher than 30%. Recognition that the tyrosine residues do not move to the interior of the protein could suggest that tryptophan residues do. The two other ratios, A_{292}/A_{274} and A_{292}/A_{254} showed a decrease for a concentration of acid near 30%. Both suggest a greater exposure of the tyrosine or a lower exposure of the tryptophan residues to the mobile phase.

Second-derivative spectroscopy. One major inconvenience in the use of absorbance ratios is the overlapping of the tryptophan band with the tyrosine and phenylalanine spectra. Second-derivative spectroscopy has been shown to be an effective tool for differentiating between contributions of each amino acid residue to the UV spectra of proteins[24]. More recently, it has been applied to the determination of

tyrosine exposure in proteins[25]. This technique has also been used to obtain information on conformational changes of proteins during chromatography[23]. The limitation of this method is that in order to obtain satisfactory precision in the observation of tyrosine movement, it is necessary that the protein contains twice as much tyrosine as tryptophan. Of the proteins studied in this work, OVA fulfills this requirement (Table II). Table III gives the results obtained for the measurement of the *a/b* ratio for different acid concentrations, where *a* and *b* indicate the peak-to-peak distances between the maximum at 288 nm and the minimum at 283 nm and the maximum at 296 nm and the minimum at 292 nm, respectively. This ratio has been shown to reflect the exposure of tyrosine to the surface[25]. Each value is the average of at least four measurements. The maximum difference obtained between them is also reported in this table. These data reveal a slight increase in the *a/b* ratio, which reflects an increase in the exposure of tyrosine residues to the exterior of the protein. These results are in agreement with the one obtained above in the measurement of absorbance ratios.

It is now possible to state that for acid concentrations higher than 30%, the decrease in protein retention is not due only to the higher solvophilic power of formic acid relative to water but also to various conformational states of the proteins. Spectroscopic measurements display a decrease in the A_{292}/A_{274} and A_{292}/A_{254} absorbance ratios. This observation, plus the increase in the *a/b* ratio obtained in second-derivative spectroscopy, reveals the movement of the tyrosine residues to the exterior of the protein. It is not possible to visualize the movement of any of the aliphatic amino acid residues, but it is probable that the behavior of tyrosine is representative for most of the residues containing a polar group. This largely influences the solubility of the proteins in the chromatographic eluent, and their retention. These results are consistent with the fact that high concentrations of acid are effective in the chromatography of large, hydrophobic proteins.

Refolding of proteins

A major concern in any chromatographic method is the conservation of the activity of the proteins. In RPC, the nature of the eluents causes the unfolding of polypeptides. However, it is possible for some proteins to regain their activity when the acid and organic solvent are removed. Ribonuclease A is well known for its refolding capability and OVA for its irreversible unfolding. Luiken *et al.*[9] have used

TABLE III

SECOND-DERIVATIVE SPECTROSCOPY OF OVALBUMIN (*a/b* RATIOS FOR DIFFERENT FORMIC ACID CONCENTRATIONS)

Formic acid concentration	*a/b Ratio*
10	0.79 ± 0.06
30	0.71 ± 0.04
60	0.84 ± 0.04
80	0.94 ± 0.04

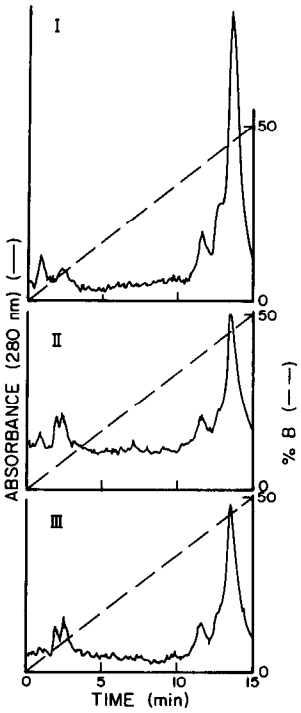

Fig. 6. Chromatographic pattern of ribonuclease A in cation-exchange chromatography. (I) native enzyme, (II) after RPC, 0.1% formic acid, (III) after RPC with 80% formic acid. For chromatographic conditions see Materials and Methods section.

ion-exchange chromatography to demonstrate the structural changes observed in these proteins after RPC. The same technique has been applied in our work for proteins eluted with 0.1% and 80% aq. formic acid. The treatment applied to the proteins collected after RPC is described in the Methods section.

For ribonuclease A, the chromatographic profiles obtained in cation-exchange chromatography are the same for native enzyme and the protein eluted from the RPC column with 0.1 and 80% aq. formic acid (Fig. 6). Furthermore, the measurement of the regain of activity (*a*) for ribonuclease A was comparable for 80% and 0.1% aq. formic acid elution. A ratio of $a_{80}/a_{0.1}$ of 95% was obtained, confirming that the high concentration of formic acid does not compromise the refolding of this protein. For OVA, the precipitate obtained after lyophilization of the fractions collected in both acid concentrations could not be solubilized in phosphate buffer.

These results show that the use of harsh conditions, like 80% aq. formic acid, still allows the refolding of some proteins. Perhaps the renaturation of proteins denatured with high concentrations of formic acid will not be more difficult than that of proteins denatured with 0.1% TFA.

Chromatography of IgG

IgG is a large protein (150 kD) whose analysis by RPC is difficult due to its

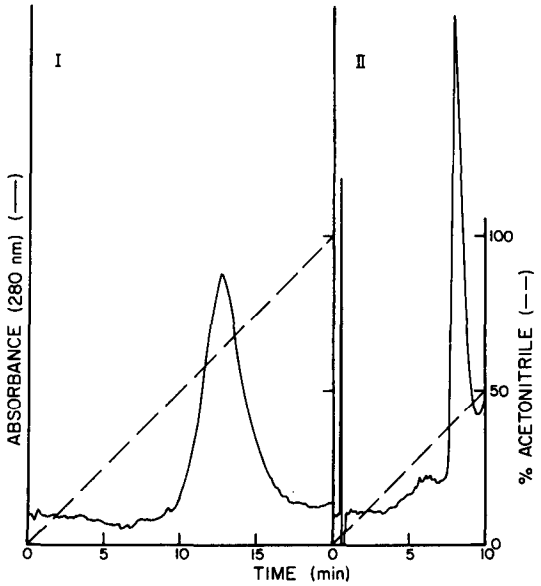

Fig. 7. Chromatographic pattern of IgG with (I) 0.1% formic acid, (II) 80% formic acid and a 20-min linear gradient of acetonitrile.

hydrophobicity. Usually, standard RPC elution with 0.1% TFA or 0.1% formic acid in acetonitrile results in a broad peak, as shown in Fig. 7. The improvement in the efficiency at 80% aq. formic acid is obvious.

An important consideration in any chromatographic method is solute recovery. Recovery of IgG from RPC columns with 0.1% TFA–acetonitrile can be very low. It was of interest to determine whether the improvement in peak shape was accompanied by a better protein recovery. Table IV reveals that the recovery is comparable for 0.1 and 80% aq. formic acid. However, better peak shape, and no additional loss in recovery make the use of 80% of formic acid a useful procedure in the RPC of IgG.

Stability of the chromatographic sorbent

All results presented in this paper have been obtained with poly(styrene–divinylbenzene). The use of high concentrations of acid had little effect on the chroma-

TABLE IV

RECOVERY OF RABBIT IgG FOR VARIOUS FORMIC ACID CONCENTRATIONS

Formic acid concentration	*Recovery (%)*
0.1	73
60	75
80	69

tographic performance of the column. Retention and peak shape for proteins were identical before and after this study[28].

CONCLUSIONS

Our results suggest that the role of acid in reversed-phase chromatography of proteins is dependent on its concentration. At concentrations of less than 10%, the acid acts both as a denaturant and as an amine pairing agent. This induces an increase in the retention of the protein when the acid concentration increases. At higher concentrations of acid, ranging from 10–30%, further denaturation of proteins occurs, with a migration of hydrophobic residues from the interior of the protein to more accessible regions on the exterior of the structure. The overall effect of this migration is an increase of the hydrophobicity of the solute and, consequently, an increase in retention. At concentrations of acid greater than 30%, the solution becomes so polar that hydrophobic residues begin to migrate into the interior of some highly denatured structure. Both, absorbance ratio measurements and second derivatives, have shown that tyrosine residues are more exposed to the exterior of the protein when the acid concentration increases. Accumulation of hydrophilic amino acids on the exterior of the molecule, plus the higher solvophilic power of formic acid compared to water result in a decrease in proteins retention. In general, this means that alterations in the hydrophobic amino acids of a protein will be most easily detected at intermediate acid concentrations; *i.e.*, 10–30%. These studies also suggest that, unless a polypeptide contains hydrophilic amino acids that may be forced to the exterior, high concentrations of acid will not be particularly effective in increasing solubility.

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