REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF VIRUS PROTEINS AND OTHER LARGE HYDROPHOBIC PROTEINS IN FORMIC ACID CONTAINING SOLVENTS

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SUMMARY

The excellent dissolving capacity of formic acid together with a propanol-2 gradient is utilized in a new system for reversed-phase high-performance liquid chromatographic separation of poliovirus polypeptides and a variety of large proteins. Differences in elution characteristics were detected between reduced and non-reduced proteins containing disulphide bridges as well as proteins modified at cysteinyl residues. The retention coefficients of single amino acids were used to calculate those of proteins. The correlation of calculated coefficients with actual retention times indicates that some proteins are bound via their full, unfolded length to the reversed-phase support, whereas others partly preserved their secondary structure. Treatment of proteins with sodium dodecyl sulphate prior to injection dissociates these structural elements and leads to an increase in retention times.

The high resolution of the system described should be applicable to the isolation and characterization of components of mixtures of proteins, particularly those of water-insoluble proteins of membranes or viruses, on the analytical and semi-preparative scales.

INTRODUCTION

During the last 6 years there have been numerous publications on reversed-phase high-performance liquid chromatography (RP-HPLC) of amino acids, peptides and low-molecular-weight polypeptides (MW < 5000) which demonstrate the enormous resolution power for this class of substances[1-36] (for reviews see refs. 13, 23, 24, 31). Several authors described the successful use of RP-HPLC for separating closely related peptides differing by one in the number of amino acid residues or by one or more in the kind of amino acid residue, including optical isomers and sequences of amino acid residues[37,38]. RP-HPLC has become a well established method for peptide separation and this technique is today one of the best methods for the separation of peptide mixtures generated by enzymatic (*e.g.*, tryptic) or chemical cleavage of proteins.

There have been fewer reports on RP-HPLC separation of larger polypeptides (MW = 10–20 kD). Examples are the separation of several hemoglobin variants[39–41], human collagen types[42], cytochrome c from several species[43] and large fragments of BrCN cleavages of hemoglobin[44], cytochrome $b5$[45], bacteriorhodopsin[46] and collagen[47,48].

Mönch and Dehnen[49] showed for the first time that proteins like bovine serum albumin may be eluted from reversed-phase columns. Later, further chromatographic conditions were introduced for the separation of larger polypeptides, however, there are only a few examples of proteins separated by RP-HPLC[13,14,50–54]. The specific difficulties in RP-HPLC of larger polypeptides are connected with the low solubility of proteins in the elution systems used.

Acetonitrile, due to its low viscosity and UV-absorption[55], together with various buffer systems is frequently used in RP-HPLC of peptides, but is less suitable for the separation of large polypeptides on account of the low solubility of proteins in the high concentrations of acetonitrile which would be necessary for elution. Propanol-1[6,13,22,44,54] or propanol-2[44,49,52] is more suitable as an organic modifier in reversed-phase protein separation. Proteins are more soluble in these alcohols and moreover the concentration for elution may be decreased due to their higher elution strength[44,56]. The addition to eluents of chaotropic salts (*e.g.*, $NaClO_4$)[41,57,58], ion-pairing substances (tri- or tetraalkylammonium phosphate)[7–9,15,36] and high concentrations of pyridine-formate or -acetate[6,13,54,56,59] improved the elution and separation of proteins, but there are many proteins of highly hydrophobic nature which cannot be eluted from reversed-phase columns by these solvents.

Many structural proteins of viruses are highly hydrophobic in nature and therefore difficult to handle. One of the most hydrophobic viruses is poliovirus. Poliovirus has two additional properties which make it ideal as a difficult model for separation procedures: it is a very compact and therefore hard to dissociate, and it contains four proteins three of which have very similar molecular weights[60,61].

We have tested several systems, including those of Gerber *et al.*[46], Takagaki *et al.*[45] and Mahoney and Hermodson[44] which are supposed to be suitable for elution of large hydrophobic peptides, to separate the four structural polypeptides of poliovirus, VP 1, 2, 3 and 4, on a C_8 or C_{18} reversed phase, but could never obtain an elution. The polypeptides were adsorbed by the column.

From our experience of the separation of these virus polypeptides by polyacrylamide electrophoresis in highly concentrated formic acid[62], we have developed a RP-HPLC elution system with a high proportion of formic acid to separate highly hydrophobic, large polypeptides. We present here a general method for separating and purifying particularly water-insoluble proteins of cell membranes and viruses by RP-HPLC with a solvent that dissolves all proteins. This solvent is volatile, which is important for easy sample recovery without desalting procedures. The system is applicable to the separation of mixtures of proteins on both analytical and preparative scales.

EXPERIMENTAL

Chromatography

HPLC experiments were carried with equipment consisting of two HPLC

pumps FR-30 (Type 5200), a gradient former (Type 9100) with mixing chamber and a variable wavelength monitor (Type 8700) set to 278 nm, all from Knauer (Berlin, G.F.R.) and a chart recorder, Beckman Instruments (München, G.F.R.). The RP-column was an Aquapore RP-300 (particle size 10 μm) from Brownlee Labs. procured by Kontron Analytic (München, G.F.R.). A Rheodyne syringe-loading sample injector (Type 2120) with a 100-μl sample loop was connected to the column which was jacketed and thermostatted at 22°C by a water-bath (Type RM3), Lauda (Tauber, G.F.R.). The eluents were prepared by mixing pure solvents (water, propanol-2, butanol-1, formic acid or acetic acid) by volume as indicated, filtered through a 0.2-μm Millipore filter and degassed under vacuum. The gradient was started immediately upon sample introduction and proceeded for a total of 60 min at a flow-rate of 1 ml/min. Elution profiles of the gradient are shown in the figures. Fractions (200–800 μl) were collected manually on the basis of detector signal, lyophilized in a cautiously evacuated desiccator over KOH pellets and prepared for rechromatography or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis

A 7.5–25 % linear gradient slab gel (1 mm thick) with 3.3 % C* according to Lambin *et al.*[63] and a discontinuous Tris–glycine buffer system[64] was used. Samples were heated to 100°C for 5 min in buffer containing 1 % SDS and 1 % 2-mercaptoethanol or dithiothreitol (DTE). Electrophoresis was performed for 16 h with 125 V at 6–8°C, then gels were stained with Serva Blau R [0.025 % in methanol–water–acetic acid (5:4:1)] and destained with 10 % acetic acid.

Materials

The following proteins and chemicals were purchased from Serva (Heidelberg, G.F.R.): ribonuclease A, cytochrome *c* (horse heart), lysozyme (hen egg), bovine serum albumin (BSA), conalbumin (bovine), soy bean trypsin inhibitor, myoglobin (whale and horse), β-lactoglobulin AB (bovine), carbonic anydrase (bovine erythrocytes), ovalbumin, acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylenediamine, mercaptoethanol, Serva Blau, R and SDS. Pure β-lactoglobulin A was a gift from Pharmacia (Freiburg, G.F.R.).

S-Aminoethyl-BSA (SAE-BSA)[65], S-β-(2-pyridylethyl)-BSA (SPE-BSA)[66], S-carboxyamido-BSA (CAM-BSA)[67] and S-sulphoethyl-BSA (SSE-BSA)[68] were prepared according to the references.

Propanol-2 (HPLC grade) and disposable extraction columns, type octadecyl, 1 ml, were from J. T. Baker (Gross Gerau, G.F.R.). Tri-*n*-butylphosphine (TBP) and 4-vinylpyridine were obtained from Fluka (Buchs, Switzerland) and sodium 2-bromoethanesulphonate from Sigma (München, G.F.R.). All other reagents were analytical grade from E. Merck (Darmstadt, G.F.R.).

The water used in HPLC experiments was deionized and purified further by pouring through a column of activated charcoal.

Poliovirus type 1, strain Mahoney, was obtained, purified and characterized as described previously[69,70] and stored in 3 M CsCl solution at -20°C.

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* The percentage concentration of bisacrylamide relative to the total concentration of acrylamide and bisacrylamide per 100 ml solution.

TABLE I

PHYSICO-CHEMICAL CHARACTERIZATION OF POLIOVIRUS POLYPEPTIDES

Polypeptide	Molecular* weight	Amino acid* units	Apparent** isoelectric point	N-terminal*,*** amino acid	Relative [§] hydrophobicity	Molar theor. [§§] extinction coefficient
VP 1	33,521	302	8.1	Gly	1.25	44,580
VP 2	29,985	271	6.4	Ser	1.19	49,680
VP 3	26,410	238	6.0	Gly	1.46	28,440
VP 4	7385	68	7.3	Blocked(?)	0.824	5520

    * Calculated from RNA sequence[71].
   ** From ref. 74.
 *** From ref. 77.
   [§] Calculated from sequence[71] by the method of Bull and Breese[76].
  [§§] Calculated from sequence[71] by the method of Wetlaufer[78].

RESULTS AND DISCUSSION

Separation of poliovirus polypeptides

Poliovirus, a member of the picornavirus group, contains one molecule of single-stranded RNA, 7440 nucleotides long[71,72] and 60 copies of four capsid polypeptides VP 1–4[60] (for physico-chemical characteristics see Table I). Poliovirus polypeptides have been separated by SDS-PAGE[60,61] and isoelectric focusing[73–75], but these techniques are time-consuming and not always suitable for preparation of pure polypeptides used directly for chemical analysis.

In our first experiments by reversed-phase HPLC we established that poliovirus polypeptides are retained by the column. Neither the elution system of Mönch and Dehnen[49] (propanol-2–phosphoric acid plus amounts of ethylene glycol monomethyl ether) nor those of Mahoney and Hermodsen[44] (propanol-1 and trifluoroacetic acid) and Takagaki et al.[45] and Gerber et al.[46] (ethanol–5 % formic acid) were appropriate for poliovirus polypeptides. In preliminary experiments we used disposable extraction columns filled with 200 mg octadecyl-modified silica to obtain a solvent mixture that solubilized the polypeptides adsorbed on the reversed phase. We have previously employed the dissolving capacity of formic acid for a polyacrylamide gel electrophoresis system. Therefore a high proportion of formic acid in the elution solution was expected to be useful for eluting the polypeptides. In a series of experiments the small disposable columns were loaded with polypeptides, and then washed with mixtures of formic acid, propanol-2 and water, the composition of which was varied stepwise. The effluents were fractionated, dried and analysed by SDS-PAGE. In this way the optimum elution conditions could be determined.

A concentration of 50–60 % (v/v) formic acid in both aqueous (solution A) and alcoholic (solution B) solution is necessary for effective elution of the virus polypeptides. The RP-HPLC column used (Aquapore RP-300) was prepacked with a wide-pore silica support of 30 nm average pore diameter, because it had been shown that an increase to this pore diameter from that used previously (6–10 nm) markedly increased the resolution of macromolecules, while at the same time retention times were decreased[42,47,79–82]. With a linear gradient from 10 to 20 % propanol-2 in 60 %
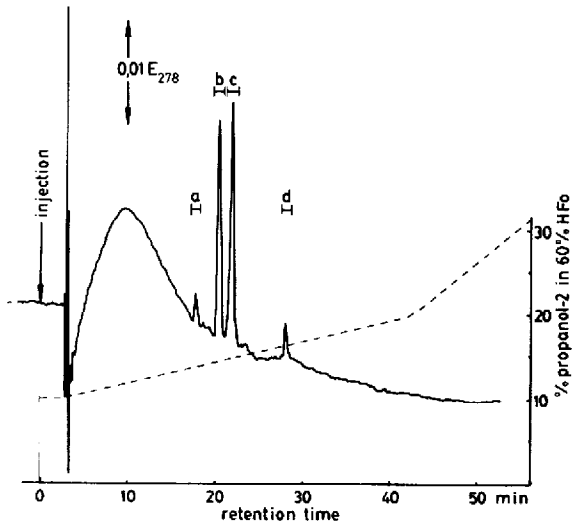
Fig. 1. HPLC separation of 20 µg poliovirus, type 1, strain Mahoney, on reversed-phase column of Aquapore RP-300. The sample contained in 3 M CsCl was injected without pretreatment. Fractions a–d, marked by bars, were collected and applied to SDS-PAGE. a = VP 4; b = VP 1; c = VP 2; d = VP 3. Elution conditions: 60 % formic acid (HFo) with a gradient of propanol-2 as indicated by the dashed line; 1 ml/min; 22°C.

formic acid we obtained a satisfactory separation of the four virus polypeptides as shown in Fig. 1. As expected from values of relative hydrophobicity (Table I), VP 4 (peak a) was eluted from the column first, followed by VP 1 (peak b) and 2 (peak c) close to one another but in inverse sequence and finally VP 3 (peak d). This order of elution and the purity of the fractionated polypeptides were checked and confirmed by SDS-PAGE (Fig. 2). From the amino acid composition deduced from the RNA sequence[71] we calculated the extinction coefficients of poliovirus polypeptides by the method of Wetlaufer[78] (Table I). On the basis of these coefficients, the injected virus amount and peak area, a nearly quantitative recovery was obtained of the poly-peptides VP 4, 1 and 2, whereas VP 3 was obtained only with a yield of about 30 %. The same result was obtained by appraising the intensities of stained protein spots on SDS-gels when equivalent amounts of the fractionated polypeptides were applied. In several experiments the eluent composition was modified to enhance the elution power, using higher concentrations of formic acid or butanol-1 instead of propanol-2. This decreased the retention times of the polypeptides but did not increase the yield of VP 3.

When formic acid was partly or completely replaced by acetic acid, a decrease in retention times of polypeptides was also observed, however, the peak widths in-creased to an extent which caused a deterioration of the resolution. Both the decrease in resolution and the increase of back-pressure were caused by the higher viscosity of acetic acid in comparison to that of formic acid. Moreover, the yields of fractionated polypeptides were reduced. Therefore, all subsequent experiments were done with formic acid.

We have also tested the influence of sample pretreatment and injection on
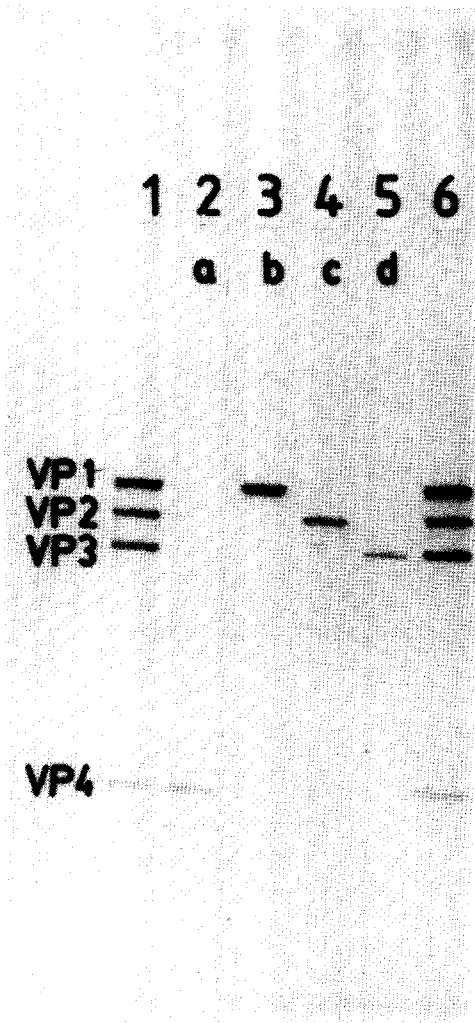
Fig. 2. SDS-polyacrylamide gel electrophoresis of poliovirus polypeptides (type 1, strain Mahoney) separated by RP-HPLC. Aliquots of the fractions a–d from the separation shown in Fig. 1 were analysed in lanes 2–5. SDS-dissociated polioviruses appeared together in lanes 1 and 6.

retention and recovery of the polypeptides. Dissociation of the poliovirus in highly concentrated formic acid prior to application on the column is not necessary: there is no difference in retention and recovery in comparison to direct injection of a virus solution kept in 3 M CsCl. This shows that virus particles are immediately dissociated on the top of the column. For all subsequent experiments direct injection was used. (Syringe and injection valve were rinsed with 80 % formic acid after each injection to destroy infections material.) The wide peak seen in the initial part of the chromatogram is caused by the RNA. The collected fraction showed an intense absorption at 260 nm. This peak does not occur if the virus sample is pretreated with ribonuclease in aqueous solution at 50°C for 30 min followed by reduction with DTE.

Influence of secondary structure on separation of proteins

For further studies of the properties and suitability of the new chromatographic system for protein separation, we used several, easily available proteins with a wide range of molecular weight from 11,700 (cytochrome *c*) to 86,000 (conalbumin) and very different hydrophobicities.

An excellent separation of a mixture of twelve proteins dissolved in water or 5 % acetic acid without reducing agents was achieved if they were chromatographed under conditions nearly identical to those applied for virus polypeptides (Fig. 3, lower chromatogram). The elution points of poliovirus polypeptides are marked by arrows. The heme from myoglobin is split off by the acidic conditions of the eluent[41] and is eluted close to cytochrome *c*. The heme-free myoglobin molecules from whale and horse are well separated. *β*-Lactoglobulin AB, a commercial mixture of the variants A and B, is also well separated, although the variants differ only by two amino acids[83], but *β*-lactoglobulin B and myoglobin from horse are co-eluted.

Previously we have shown that poliovirus polypeptides contain only SH groups but no disulphide bonds[84]. To study the behaviour of proteins containing SH groups, the protein mixture was reduced either with DTE at pH = 7.5 in 0.01 *M* phosphate buffer or with tri-*n*-butylphosphine (TBP) in 5 % acetic acid[85].
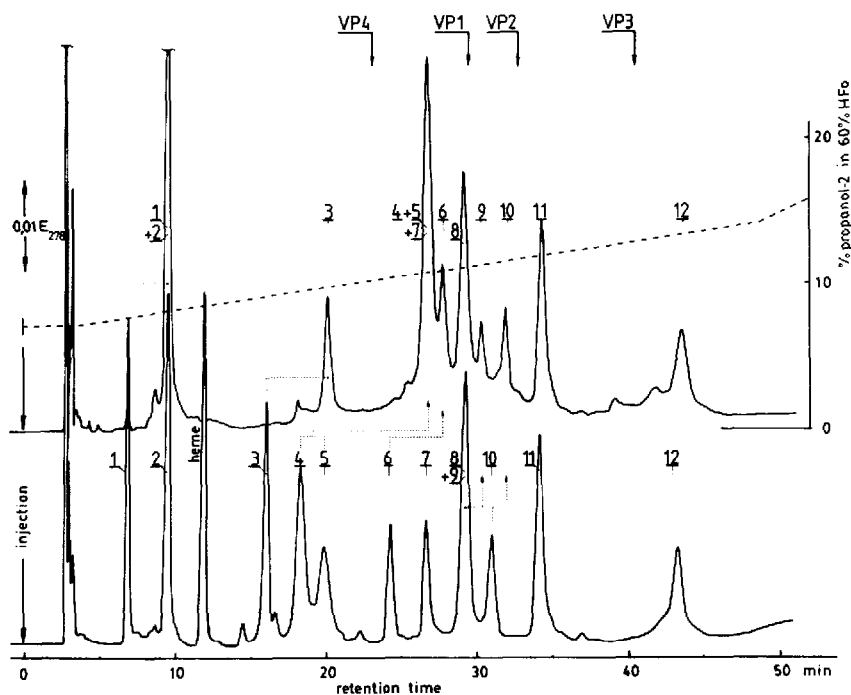

Fig. 3. Comparison of the chromatographic behaviour of reduced (upper chromatogram) and non-reduced (lower chromatogram) proteins in the formic acid/reversed-phase system. The protein mixture dissolved in 5 % acetic acid consists of 0.5–1 mg/ml each of: 1 = ribonuclease A; 2 = cytochrome *c*; 3 = lysozyme; 4 = BSA; 5 = conalbumin; 6 = trypsin inhibitor; 7 = whale myoglobin; 8 = horse myoglobin; 9 + 10 = *β*-lactoglobulin A and B; 11 = carbonic anhydrase; 12 = ovalbumin. For reduction, 10 *μ*l/ml TBP (diluted with 90 *μ*l propanol-2) were added and incubated for 2 h at 37°C. Shifts of the elution positions after reduction are marked by dotted lines. Elution conditions as in Fig. 1.
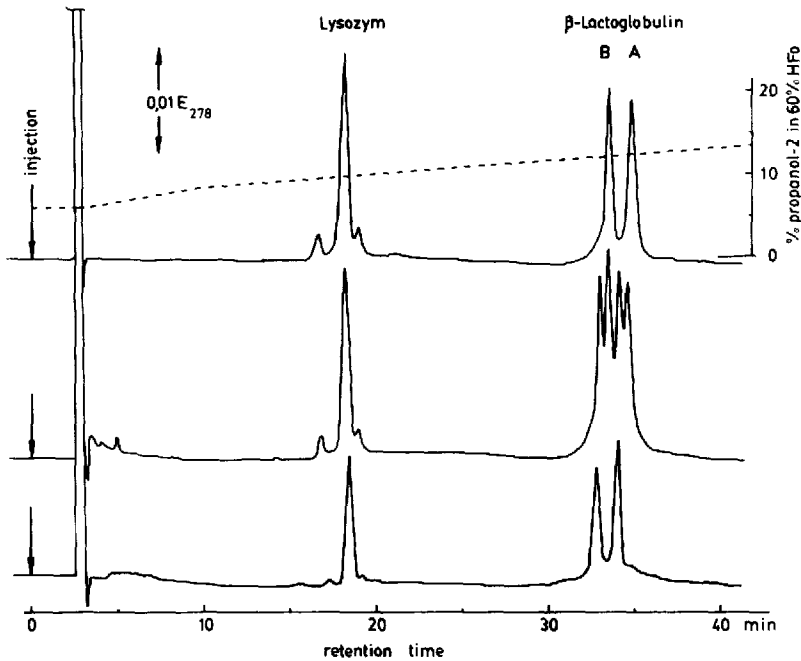
Fig. 4. Chromatography of 25–30 μg of a commercially available mixture of β-lactoglobulin A and B, non-reduced (bottom chromatogram), reduced with TBP in 5 % acetic acid (top chromatogram) and a mixture of both reduced and non-reduced β-lactoglobulin AB (middle chromatogram). Lysozyme was used as internal standard. Conditions as in Fig. 1.

During reduction in neutral or slightly alkaline aqueous solution some proteins became insoluble and precipitated, and had to be redissolved in 20 % formic acid before injection. The upper chromatogram in Fig. 3 shows the separation of the reduced protein mixture. Shifts in the retention of proteins containing disulphide bonds (ribonuclease, lysozyme, BSA, conalbumin, trypsin inhibitor, β-lactoglobulin), depending on the secondary structure, i.e., the number and position of intramolecular disulphide bonds. The shift in the elution position of the trypsin inhibitor containing two disulphide bridges is clearly visible. In contrast, the shift of β-lactoglobulin A or B, which also contains two disulphide bridges, is barely detectable.

Fig. 4 shows chromatograms of the reduced (top) and non-reduced (bottom) β-lactoglobulin A and B and of a mixture of both (middle). The excellent separation of these two very closely related proteins is seen, and even four peaks can be detected in the mixture From the above differences between the reduced and non-reduced proteins it is deduced that the interaction between the reversed phase and proteins after reduction of disulphide bonds is stronger than that with the untreated proteins. This means that the area of contact of the reduced proteins has been increased by more extensive unfolding.

However, it is not clear whether protonation and solvation by formic acid would lead to completely unfolded protein molecules if there were no stabilization by disulphide bonds. Formic acid in high concentration (up to 100 %) may elute only a few peptides and polypeptides from reversed-phase columns if organic solvents are

omitted. This indicates that it only partly dissociates hydrophobically bonded structures and it may be that secondary structures, stabilized by hydrophobic forces, are preserved.

The question thus arises as to whether the reduced proteins are completely stretched and interact with their whole length with the reversed phase or whether secondary structures are preserved. We have applied the correlation of hydrophobic parameters to retention times introduced by Meek[57], Meek and Rossetti[58] and Wilson et al.[56,86] for peptides, and the hydrophobic fragmental constants tabulated by Rekker[87], to calculate the retention behaviour or reduced proteins on RP-HPLC. Molnar and Schoeneshoefer[88] studied polypeptides whose low retention times (lower than calculated) were explained by the assumption that a secondary structure was stabilized by intramolecular hydrophobic interactions. For our calculation we used the improved retention coefficients of amino acids given by Meek and Rossetti[58], although their chromatographic conditions were different from ours. Therefore, although the calculated retention times cannot be expected to be in numerical agreement with the observed ones, a correlation between them should be found. Fig. 5 shows a plot of the actual retention times *versus* retention coefficients of some reduced proteins, calculated by summing the coefficients of the constituent amino acids (obtained from sequence data [83,89–92]).

To a first approximation there is a linear correlation (correlation coefficient $R = 0.97$, if BSA, lysozyme and cytochrome are not taken into account) between the total hydrophobicity of proteins and their elution points in our chromatographic system, although a few considerable deviations are observed. A similar graph was obtained by using the hydrophobic fragmental constants to calculate the total hydrophobicity of the proteins. This demonstrates that in two differing analytical systems the hydrophobicity of amino acids is independent of the solvent composition, when
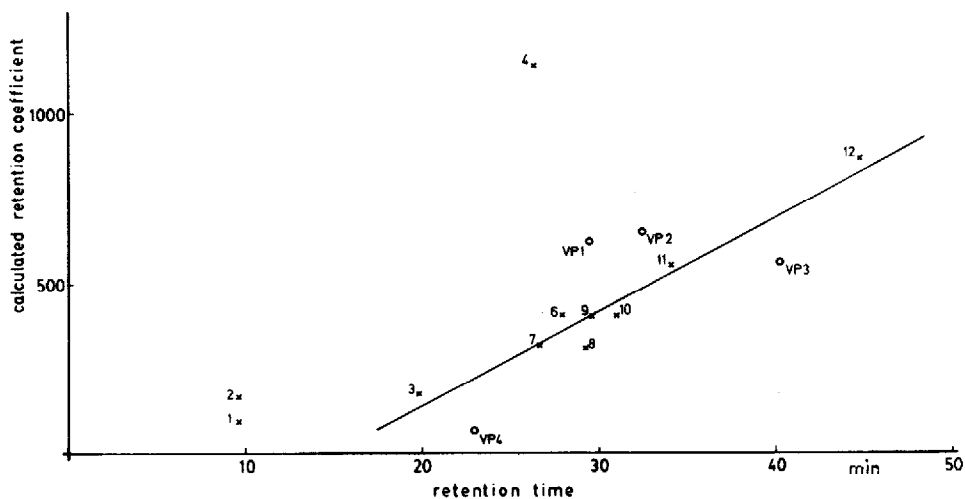

Fig. 5. Correlation of actual retention times *versus* retention coefficients of proteins calculated by summation of the coefficients of amino acid residues[58] (phosphate system). Numbers adjacent to the data points indicate the proteins listed in Fig. 3. The corresponding values of poliovirus polypeptides are also noted.
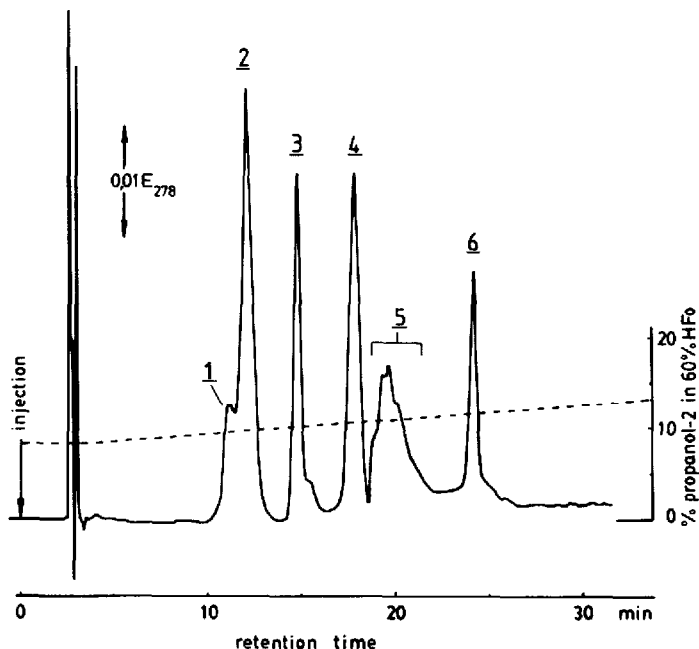
Fig. 6. Separation of BSA derivatives with different modifications at cysteinyl residues: 1 = S-aminoethyl-BSA; 2 = S-pyridylethyl-BSA; 3 = non-reduced BSA; 4 = S-carboxyamidomethyl-BSA; 5 = S-sulphoethyl-BSA; 6 = reduced BSA. About 25 µg were injected. Conditions as in Fig. 1.

the reference system is identical (glycine). The values of hydrophobicity undergo only a relative displacement by changing from aqueous–alcoholic elution systems to those containing a high concentration of formic acid. BSA, cytochrome c and ribonuclease as well as the virus polypeptides VP 1 and 2 are eluted much earlier, but VP 3 and 4 are retarded to a greater extent, than expected.

In order to determine whether the anomalous elution behaviour of BSA is based on incomplete reduction, BSA was treated with DTE under denaturating conditions in 8 M urea or 6 M guanidine hydrochloride and then alkylated to protect cysteinyl residues. Four differently modified BSA preparations were obtained: S-aminoethyl-BSA and S-pyridylethyl-BSA with basic protection groups, S-carboxamidomethyl-BSA with a neutral and S-sulphoethyl-BSA with an acidic protection residue. These four derivatives are insoluble in water but soluble in 10 % formic acid. Fig. 6 shows their chromatographic behaviour in our system in comparison with reduced but unmodified and with non-reduced BSA. The change in elution behaviour observed after modification of thiol groups indicates that the intrinsic charge of the protein influences binding to the reversed phase. The total hydrophobicity decreased with increasing basicity of the substituents at the cysteine residues. In the acidic solvent used, the protonation of basic amino or pyridyl groups promotes the elution, therefore SAE-BSA and SPE-BSA are eluted earlier than CAM-BSA (neutral substituent) and SSE-BSA (acidic substituent). The broad peak of SSE-BSA is probably caused by an incomplete reaction of BSA with the 2-bromoethylsulphonate.

All of the modified BSA derivatives were eluted earlier than reduced BSA,

therefore we assume that reduction was in fact complete and the anomalous elution characteristics of BSA are a result of a partly folded secondary structure which is stabilized by intrachain interactions not dissociable by formic acid. It is known that some proteins —also those like ribonuclease— which contain disulphide bonds can return to their native states even when the disulphide bonds are reduced simultaneously with denaturation. Therefore it can be deduced that proteins which elute earlier than expected with respect to their total hydrophobicity, such as ribonuclease, cytochrome c, BSA and the poliovirus polypeptides VP 1 and 2, contain intramolecular folded domains stabilized by hydrophobic forces that cannot be dissociated by elution solvents.

While poliovirus particles are dissociated to RNA and polypeptides immediately after injection on the column, the secondary structure of VP 1 and 2 is preserved at least to some extent during the residence time in the column. When the polypeptides are stored for several hours in the elution solvent a change in the structure and interchain aggregation must occur, because the once separated polypeptides were then retained by the column upon rechromatography. SDS dissociates all polypeptides separated by our system, *i.e.*, also aggregated polypeptides, as has been shown by their subsequent separation by SDS-PAGE. Here we find a remarkable difference between poliovirus polypeptides and the other proteins tested. Separated virus polypeptides could not be rechromatographed at all or only with very low yields, whereas all the other proteins used were quantitatively rechromatographed by repeated injection and elution after drying and redissolving the fractionated mixture. The only exceptions were reduced BSA and ovalbumin which were rechromatographed with a loss of about 20–40%.

We explain the decelerated elution of a few proteins —especially VP 4 and 3— in comparison to their calculated hydrophobicities by a stronger interaction with the support. This could be caused by oligomerization which leads to molecules with increased total hydrophobicities. The low chromatographic yield of VP 3 and the unusual retardation (monomeric VP 3 should be eluted prior to VP 1) might be an indication of oligomerization. The aberrant migration of VP 4 in formic acid polyacrylamide gel electrophoresis[62] could also be explained by oligomerization.

SDS-containing protein samples

Formic acid is an excellent solvent for proteins, but some chemical reactions are difficult to perform in acidic media. Therefore SDS is often used to dissolve viral or membrane proteins in order to perform reactions in neutral, aqueous media. We have therefore tested protein–SDS complexes with regard to their chromatographic behaviour on reversed-phase columns. Solutions containing sufficient amounts of SDS and reducing agents effectively solubilize proteins by simultaneous alteration of their conformations and generation of uniformly shaped protein–SDS complexes. Such alterations in conformation, and the coating of the proteins with the hydrophobic SDS residues, were expected to affect the elution behaviour of the proteins. In our formic acid-containing system, polypeptides which stayed in the column only for a short period of time (ribonuclease and cytochrome c) were eluted with some delay as broad peaks. The other proteins tested were eluted nearly unchanged as small peaks with the exception of β-lactoglobulin B and the virus polypeptides VP 1 and 2. The separation between β-lactoglobulin A and B was lost, both co-eluting at the

position of the reduced A variant (without SDS treatment). VP 1 and 2 were more strongly retarded, co-eluting near the position of VP 3.

This different behaviour of SDS-treated samples can be explained in the following way. Amons and Schrier[93] reported the dissociation of protein–SDS complexes by propionic acid–formic acid–water (2:1:2). Hence we may infer that also the eluent formic acid–propanol-2 will cause dissociation of SDS–protein complexes into monomeric SDS and free protein on top of the column. Changes in the retention of β-lactoglobulin as well as of VP 1 and 2 seem to be the result of an alteration of their secondary structure. Treatment with SDS dissociates all intrachain foldings stabilized by hydrophobic forces, including those which are not affected by formic acid. After removal of the SDS, the protein does not return to the conformation obtained without SDS treatment. The elution positions are now in good agreement with the calculated elution coefficients. The relatively broad peaks of ribonuclease and cytochrome c treated with SDS support the idea that an inhomogeneous population of molecules is generated on the column due to either incomplete dissociation of SDS and/or different secondary structures of the protein molecules.

CONCLUSIONS

The present RP-HPLC method with solvents containing high proportions of formic acid is generally applicable to analytical and preparative separations of water-soluble as well as of water-insoluble and hydrophobic proteins. The strong dissolving capacity and the powerful elution strength of the solvent systems described overcome the problems of insolubility of virus proteins and membrane proteins as well as of chemically modified proteins in aqueous detergent-free systems.

For the recovery of the separated proteins, the eluent formic acid–porpanol-2 may be removed without residue by simple evaporation. Thereby pure polypeptides are quickly obtained which are suitable for chemical analysis. The system described is also applicable to the detection of intrachain disulphide bridges.

REFERENCES

1 R. Burgus and J. Rivier, in A. Loffett (Editor), *Peptides 1976, Proc. 14th Eur. Peptide Symp.*, University of Brussels, Belgium, 1976, p. 85.
2 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
3 K. S. Axelsen and S. H. Vogelsang, *J. Chromatogr.*, 140 (1977) 174.
4 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
5 W. Mönch and W. Dehnen, *J. Chromatogr.*, 140 (1977) 260.
6 M. Rubinstein, S. Stein, L. D. Gerber and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 3052.
7 J. E. Revier, *J. Liquid Chromatogr.*, 1 (1978) 343.
8 W. S. Hancock, C. A. Bishop, R. L. Prestidge and M. T. W. Hearn, *Anal. Biochem.*, 89 (1978) 203.

9 W. S. Hancock, C. A. Bishop, R. L. Prestidge and D. R. K. Harding, *Science*, 200 (1978) 1168.
10 B. Larsen, V. Viswanatha, S. Y. Chang and V. J. Hruby, *J. Chromatogr. Sci.*, 16 (1978) 207.
11 M. T. W. Hearn, W. S. Hancock, J. G. R. Hunell, R. J. Fleming and B. Kemp, *J. Liquid Chromatogr.*, 2 (1979) 919.
12 M. T. W. Hearn, B. Grego and W. S. Hancock, *J. Chromatogr.*, 185 (1979) 429.
13 M. Rubinstein, *Anal. Biochem.*, 98 (1979) 1.
14 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
15 M. E. F. Biemond, W. A. Sipman and J. Olivié, *J. Liquid Chromatogr.*, 2 (1979) 1407.
16 S. Terabe, R. Konaka and K. Inouye, *J. Chromatogr.*, 172 (1979) 163.
17 S. Terabe, R. Konaka and J. Shoji, *J. Chromatogr.*, 173 (1979) 313.
18 J. Shoji, J. Kato, S. Terabe and R. Konaka, *J. Antibiot.*, 32 (1979) 313.
19 B. T. Busch, J. H. Frenz, W. R. Melander, Cs. Horváth, A. R. Cashmore, R. N. Dryer, J. O. Knipe, J. K. Coward and J. R. Bertino, *J. Chromatogr.*, 168 (1979) 343.
20 M. T. W. Hearn and W. S. Hancock, in G. L. Hawk (Editor), *Biological/Biochemical Application of Liquid Chromatography*, Marcel Dekker, New York, 1979, p. 243.
21 W. A. Schroeder, J. B. Shelton, J. R. Shelton and D. Powars, *J. Chromatogr.*, 174 (1979) 385.
22 M. Rubinstein, G. Chen-Kiang, S. Stein and S. Udenfriend, *Anal. Biochem.*, 95 (1979) 117.
23 M. T. W. Hearn, *J. Liquid Chromatogr.*, 3 (1980) 1255.
24 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
25 K. Krummen, *J. Liquid Chromatogr.*, 3 (1980) 1243.
26 D. D. Blevius, M. F. Burke and V. J. Hruby, *Anal. Biochem.*, 52 (1980) 420.
27 J. F. M. Kinkel, G. Heuver and J. C. Kraak, *Chromatographia*, 13 (1980) 145.
28 H. Kratzin, C. Y. Yang, T. U. Krusche and N. Hilschmann, *Hoppe Seyler's Z. Physiol. Chem.*, 361 (1980) 1591.
29 J. Sugihara, T. Imamura, T. Imoto and T. Yanase, *Biochim. Biophys. Acta*, 669 (1981) 105.
30 T. Imoto and K. Okazaki, *J. Biochem. (Tokyo)*, 89 (1981) 437.
31 W. A. Schroeder, J. B. Shelton and J. R. Shelton, in S. H. Hanash (Editor), *Proceedings of the Workshop on Recent Advances in Hemoglobin Analysis, Ann Arbor, 1980*, A. R. Liss, New York, 1981.
32 L. Haeffner-Gormley, N. H. Poludniak and D. B. Wetlaufer, *J. Chromatogr.*, 214 (1981) 185.
33 C. Y. Yang, E. Pauly, H. Kratzin and N. Hilschmann, *Hoppe Seyler's Z. Physiol. Chem.*, 362 (1971) 1131.
34 P. Böhlen and G. Kleeman, *J. Chromatogr.*, 205 (1981) 65.
35 W. S. Hancock, J. D. Capra, W. A. Bradley and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 59.
36 G. Vigh, Z. Varga-Puchony, J. Hlavay and E. Papp-Hites, *J. Chromatogr.*, 236 (1982) 51.
37 E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, 50 (1978) 1353.
38 C. Hunter, K. Sugden and J. G. Lloyd-Jones, *J. Liquid Chromatogr.*, 3 (1980) 1335.
39 L. F. Congote, H. P. J. Bennett and S. Solomon, *Biochem. Biophys. Res. Commun.*, 89 (1979) 851.
40 P. E. Petrides, R. T. Jones and P. Böhlen, *Anal. Biochem.*, 105 (1980) 383.
41 J. B. Shelton, J. R. Shelton and W. A. Schroeder, *J. Liquid Chromatogr.*, 4 (1981) 1381.
42 A. Kallon, R. V. Lewis and K. D. Gibson, *Anal. Biochem.*, 110 (1981) 318.
43 S. Terabe, H. Nishi and T. Ando, *J. Chromatogr.*, 212 (1981) 295.
44 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
45 Y. Takagaki, G. E. Gerber, K. Nikei and H. G. Khorana, *J. Biol. Chem.*, 255 (1980) 1536.
46 G. E. Gerber, R. J. Anderegg, W. C. Herlihy, C. P. Gray, K. Biemann and H. G. Khorana, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 227.
47 M. van der Rest, H. P. J. Bennett, S. Solomon and F. H. Glorieux, *Biochem. J.*, 191 (1980) 253.
48 C. Black, D. M. Douglas and M. L. Tanzer, *J. Chromatogr.*, 190 (1980) 393.
49 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415.
50 R. V. Lewis, S. Stein and S. Udenfriend, *Int. J. Pept. Protein Res.*, 13 (1979) 493.
51 B. N. Jones, R. V. Lewis, S. Pääbo, K. Kojima, S. Kimura and S. Stein, *J. Liquid Chromatogr.*, 3 (1980) 1373.
52 R. A. Bardford, B. J. Sliwinski, A. C. Breyer and H. L. Rothbart, *J. Chromatogr.*, 235 (1982) 281.
53 M. P. Strickler, M. J. Gemski and B. P. Doctor, *J. Liquid Chromatogr.*, 4 (1981) 1765.
54 G. J. Hughes, K. H. Winterhalter and K. J. Wilson, in F. Lottspeich, A. Henschen and K.-P. Hupe (Editors), *High Performance Liquid Chromatography in Protein and Peptide Chemistry*, W. de Gruyter, Berlin, 1981, p. 175.
55 J. A. Riddik and W. B. Bunger, *Organic Solvents, Physical Properties and Methods of Purification*, Wiley-Interscience, New York, 1970.

56 K. J. Wilson, A. Honegger, R. P. Stölzel and G. J. Hughes, *Biochem. J.*, 199 (1981) 31.
57 J. L. Meek, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1632.
58 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
59 G. J. Hughes, K. H. Winterhalter and K. J. Wilson, *FEBS Lett.*, 108 (1979) 81.
60 R. R. Rueckert, in H. Fraenkel-Conrat and R. R. Wagner (Editors), *Comprehensive Virology*, Vol. 6, Plenum, New York, 1976, p. 131.
61 J. V. Maizel, *Methods Virol.*, 5 (1971) 179.
62 J. Heukeshoven and R. Dernick, *Electrophoresis*, 2 (1981) 91.
63 P. Lambin, D. Rochu and J. M. Fine, *Anal. Biochem.*, 74 (1976) 567.
64 J. P. Poduslo, *Anal. Biochem.*, 114 (1981) 131.
65 R. D. Cole, *Methods Enzymol.*, 11 (1967) 315.
66 J. F. Cavins and M. Friedman, *Anal. Biochem.*, 35 (1970) 489.
67 C. H. W. Hirs, *Methods Enzymol.*, 11 (1967) 199.
68 V. Niketic, J. Thomsen and K. Kristiansen, *Eur. J. Biochem.*, 46 (1974) 547.
69 R. Drzeniek and P. Bilello, *J. Gen. Virol.*, 25 (1974) 125.
70 U. Yamaguchi-Koll, K. J. Wiegers and R. Drzeniek, *J. Gen. Virol.*, 26 (1975) 307.
71 N. Kitamura, B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecah, J. J. Lee, S. v.d. Werf, C. W. Anderson and E. Wimmer, *Nature (London)*, 291 (1981) 547.
72 V. R. Racaniello and D. Baltimore, *Proc. Nat. Acad. Sci. U.S.*, 78 (1981) 4887.
73 A. Hamann and R. Drzeniek, *J. Chromatogr.*, 147 (1978) 243.
74 A. Hamann, C. Reichel, K. J. Wiegers and R. Drzeniek, *J. Gen. Virol.*, 38 (1978) 567.
75 K. J. Wiegers and R. Drzeniek, *J. Gen. Virol.*, 47 (1980) 423.
76 H. B. Bull and K. Breese, *Arch. Biochem. Biophys.*, 161 (1974) 665.
77 J. Heukeshoven and R. Dernick, unpublished results.
78 D. B. Wetlaufer, *Advan. Protein Chem.*, 17 (1962) 303.
79 C. Vanecek and F. F. Regnier, *Anal. Biochem.*, 109 (1980) 345.
80 W. Schwarz, H. Tiedemann and J. Molnar, personal communication.
81 R. V. Lewis and D. DeWald, personal communication.
82 K. J. Wilson, E. v. Wieringen, S. Klauser, M. W. Berchtold and G. J. Hughes, *J. Chromatogr.*, 237 (1982) 407.
83 G. Braunitzer, R. Chen, B. Schrank and A. Stangl, *Hoppel Seyler's Z. Physiol. Chem.*, 354 (1973) 867.
84 J. Heukeshoven and R. Dernick, *Z. Naturforsch. C.*, 36 (1981) 164.
85 U. Th. Rüegg and J. Rudinger, *Methods Enzymol.*, 47 (1977) 111.
86 K. J. Wilson, A. Honegger and G. J. Hughes, *Biochem. J.*, 199 (1981) 43.
87 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Pharmaco Chemistry Library, Vol. 1, Elsevier, Amsterdam, 1977.
88 J. Molnar and M. Schoeneshoefer, in F. Lottspeich, A. Henschen and K.-P. Hupe (Editors), *High Performance Liquid Chromatography in Protein and Peptide Chemistry*, W. de Gruyter, Berlin, 1981, p. 97.
89 R. J. Tanis, R. E. Ferrell and R. E. Tashian, *Biochim. Biophys. Acta*, 371 (1974) 534.
90 A. D. Nisbet, R. H. Saundry, A. J. G. Moir, L. A. Fothergill and J. E. Fothergill, *Eur. J. Biochem.*, 115 (1981) 335.
91 T. P. King and M. Spencer, *J. Biol. Chem.*, 245 (1970) 6134.
92 L. R. Croft, *Handbook of Protein Sequences*, Jaynson-Bruvvers Ltd., Oxford, 1973.
93 R. Amons and P. J. Schrier, *Anal. Biochem.*, 116 (1981) 439.