Journal of Chromatography, 326 (1985) 91–101
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 548

CHARACTERIZATION OF A SOLVENT SYSTEM FOR SEPARATION OF WATER-INSOLUBLE POLIOVIRUS PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JOCHEN HEUKESHOVEN* and RUDOLF DERNICK
Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20 (F.R.G.)

SUMMARY

Formic acid in high concentration is an extremely potent solvent for proteins, particularly for hydrophobic ones. 60% Formic acid, necessary for solubilization of structural polypeptides of poliovirus and other proteins, modified at the cysteines, was used together with 2-propanol or acetonitrile as organic modifier for gradient elution in reversed-phase high-performance liquid chromatography. Several reversed-phase columns were tested. In each case, polypeptides were eluted quantitatively. It was demonstrated that this solvent system, with its high proportion of formic acid, did not affect the size, hydrophobicity and charge of the separated polypeptides. By injection into rabbits of poliovirus polypeptides, obtained in high purity by chromatography in the new solvent system, monospecific antibodies were induced, the specificity of which was determined by immunoprecipitation.

INTRODUCTION

In the last few years reversed-phase high-performance liquid chromatography (RP-HPLC) has been shown to be an excellent separation technique not only for peptides, but also for soluble proteins[1–25]. Generally, the separation of water-soluble polypeptides and proteins on reversed-phase packing materials requires gradient elution with aqueous–organic solvent mixtures. The solvent systems frequently used contain low amounts of acids, such as phosphoric acid[1–6], trifluoroacetic acid[7–13], hydrochloric acid[14–16] or formic acid[17–19] with admixture of several salts to increase the solubility of polypeptides and proteins and to adjust various equilibria of these polyionic solutes. However, all of these solvent systems have proved to be useless for the separation of poliovirus structural polypeptides and their cyanogen bromide fragments, due to the exceedingly low solubility of these substances in aqueous buffers. The insolubility in aqueous solvents of proteins of higher hydrophobicity, *e.g.*, proteins of cell membranes or viruses, is one of the main problems in RP-HPLC. Solubilization of these proteins is achieved only by use of detergents or high concentrations of denaturing agents, such as 8 *M* urea or 6 *M* guanidine hydrochloride. Sometimes strong detergents, such as sodium dodecyl sulphate (SDS), are necessary. These

0021-9673/85/$03.30

solvent systems are useful in size-exclusion chromatography (SEC) but not in reversed-phase chromatography (RPC). Problems are encountered when 8 *M* urea or 6 *M* guanidine hydrochloride is used in RP-HPLC, due to the high viscosity of such solutions and to the low solubility of these denaturants in organic solvents, such as alcohols or acetonitrile.

To overcome the solubility problems of poliovirus polypeptides, we have introduced a new solvent system in RP-HPLC, which contains a high proportion of formic acid[26,27]. This system, which has excellent solvent properties, was very useful in our studies on poliovirus structural polypeptides. It might be generally applicable for the separation of water-insoluble hydrophobic proteins.

The present investigation demonstrates that this solvent system with its high concentration of formic acid has no or only a slight effect on the physico-chemical properties of the separated polypeptides, such as size, hydrophobicity and charge. Furthermore, it will be shown that the biological activity expressed as antigenicity is highly preserved.

EXPERIMENTAL

Chromatography

All chromatographic experiments were performed with a Knauer (Berlin, F.R.G.) liquid chromatographic system, consisting of two HPLC pumps FR-30, a gradient former (Type 9100) with a mixing chamber and a variable-wavelength monitor (Type 8700), set to 278 nm, and a chart recorder. A Rheodyne injector (Type 7125) was connected to the column, which was thermostatted at 25°C.

Octadecyl, diphenyl, and butyl wide-pore RP columns (250 × 4.6 nm, 5 μm) were from J. T. Baker (Gross Gerau, F.R.G.). The eluents were prepared by mixing pure solvents (water, formic acid, acetonitrile) by volume, as indicated, filtered through a 0.2-μm Millipore filter (pure cellulose) and degassed under vacuum. The gradient was started immediately after sample injection, but its action is delayed by 3 min at a flow-rate of 1 ml/min.

The elution profiles of the gradient are shown in the figures. Fractions (500–1000 μl) were collected manually on the basis of the detector signal, and put in a desiccator containing potassium hydroxide pellets which was connected to a freezing trap. By cautious evacuation with an oil pump to 0.1 mbar, the solvent was evaporated, causing the fractions to cool down by themselves. Before the fractions were completely dry, 500 μl of water were added and drying was continued. This procedure was repeated three times. The samples were then suitable for rechromatography, electrophoresis or immunization.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed either on vertical 1-mm slab gels with a linear gradient of 7.5–25% acrylamide[26,28] or on horizontal ultrathin (180-μm) homogeneous gels of 12.5% acrylamide[28]. Gels were fixed in 30% ethanol–10% acetic acid and stained by the silver-staining method described in detail elsewhere[28,30].

Isoelectric focusing (IEF) was performed in 1-mm polyacrylamide gels, covalently bound onto silane-coated glass plates (230 × 115 mm), as generally described by Radola[29] for ultrathin-layer isoelectric focusing.

The gels consisted of 5% acrylamide cross-linked by bisacrylamide (ratio 37.5:1) and contained 9 *M* urea.

Virus

Labelled and unlabelled poliovirus, Type 1, strain Mahoney, Type 2, strain MEF-1 and Type 3, strain Saukett, were grown in HeLa S_3 cells, purified and characterized as described previously[31,32], and stored in 3 *M* CsCl at −20°C.

Immunization

The polypeptides (200–250 μg of each), desiccated under vacuum were dissolved in 1 ml of 8 *M* urea containing 1 mg dithioerythritol (DTE) and 0.05 *M* sodium phosphate buffer (pH 8), mixed with complete Freund's adjuvant and injected subcutaneously into 6-month-old rabbits, according to Vaitukaitis[33].

Immunoprecipitation

The antisera obtained were tested for monospecificity with SDS-dissociated poliovirus by protein-A aided immunoprecipitation and SDS-PAGE, as recently described in detail[34].

Materials

The following materials were purchased from Serva (Heidelberg, F.R.G.): bovine serum albumin, bovine carbonic anhydrase, thermolysin, trypsinogen, soy bean trypsin inhibitor, whale myoglobin, acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED), Serva Blau R and SDS. Formic acid and acetonitrile (analytical grade) were from J. T. Baker (Gross Gerau, F.R.G.) or E. Merck (Darmstadt, F.R.G.), 4-vinylpyridine and dithioerythritol (DTE) from Fluka (Buchs, Switzerland). Molecular-weight-marker proteins were purchased from Pharmacia (Uppsala, Sweden), and ampholine (pH 3.5–10) from LKB Instruments (Gräfelfing, F.R.G.). L-[^{35}S]Methionine (600–700 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, F.R.G.). All other reagents were analytical grade from Merck. Water used in HPLC was deionized and further purified on activated charcoal columns.

RESULTS AND DISCUSSION

Poliovirus, a member of the picornaviridae, contains highly hydrophobic polypeptides. The very compact virus is hard to dissociate into its components, consisting of a RNA and 60 copies of four different polypeptides, three of which have very similar molecular weights[35] (Table I). These polypeptides were found to be exceedingly insoluble in aqueous buffers after disruption of the virus with denaturing agents.

Generally, formic acid in high concentrations is an extremely potent solvent for proteins. A concentration of 60% formic acid is necessary to dissolve all four virus polypeptides. Therefore, we employed 60% formic acid in all solvents for chromatography. Recently, we described the separation of poliovirus polypeptides on a reversed-phase column with 60% formic acid and a gradient of 2-propanol as organic modifier[26,27]. Fig. 1 shows an improved separation of the polypeptides of poliovirus, Type 1, on a C_{18} column in 60% formic acid with acetonitrile as secondary solvent.

We obtained nearly quantitative recoveries of all four polypeptides, as determined with radioactively labelled poliovirus. Surprisingly, the recovery of poliovirus polypeptides was higher in the acetonitrile system than in the 2-propanol system, although in general the solubility of proteins in 2-propanol is found to be higher than in acetonitrile[39].

The detection limit was 0.1–1 μg of protein. The amount loaded had no effect on protein recoveries[12,16]. Very low amounts as well as high amounts were completely eluted in a single gradient cycle. No adsorption and desorption in consecutive elution cycles was observed when loadings for micropreparative separations were used. A 500-μg amount of virus is not the largest amount which can be separated in a single analysis. The upper limit could not be determined because only a restricted amount of virus was available.

Bonded phase type

We have mainly used C_{18} columns to separate virus polypeptides, but other bonded phases, such as butyl- or diphenyl-modified silica are also suitable in the formic acid system. With constant elution conditions and a linear gradient of acetonitrile, we observed different retentions on C_{18}, C_4 and diphenyl columns, as shown in Fig. 2, whereas in other systems[39–41] the bonded phase did not appear to matter.

Sample application

We used different methods of sample preparation. The simplest one was to inject the virus sample, obtained by purification using caesium chloride-gradient centrifugation, directly without pretreatment. Virus dissociation and solubilization of the polypeptides occurred immediately when the virus came into contact with the mobile phase. Diluted samples were concentrated by precipitation with 20–30% formic acid or by 10% trichloroacetic acid. The precipitate was dissolved either in 70–100% formic acid or in 6 *M* guanidine hydrochloride (pH 7.5–8.0), containing 0.1–1% dithioerythritol. This solution was injected directly or after modification of the proteins by thiol reagents. The solvents used were volatile and easily removed by evaporation.

To determine whether there is any alteration of the proteins chromatographed

TABLE I

PHYSICOCHEMICAL DATA OF STRUCTURAL POLYPEPTIDES OF POLIOVIRUS, TYPE 1, STRAIN MAHONEY

Polypeptide	*Mol. weight**	*pI in 8 M urea***	*Rel. hydrophobicity****
VP1	33521	8.1	1.25
VP2	29985	6.4	1.19
VP3	26410	6.0	1.46
VP4	7385	7.3	0.82

* From RNA sequence[36].
** Ref. 37.
*** Calculated according to ref. 38.

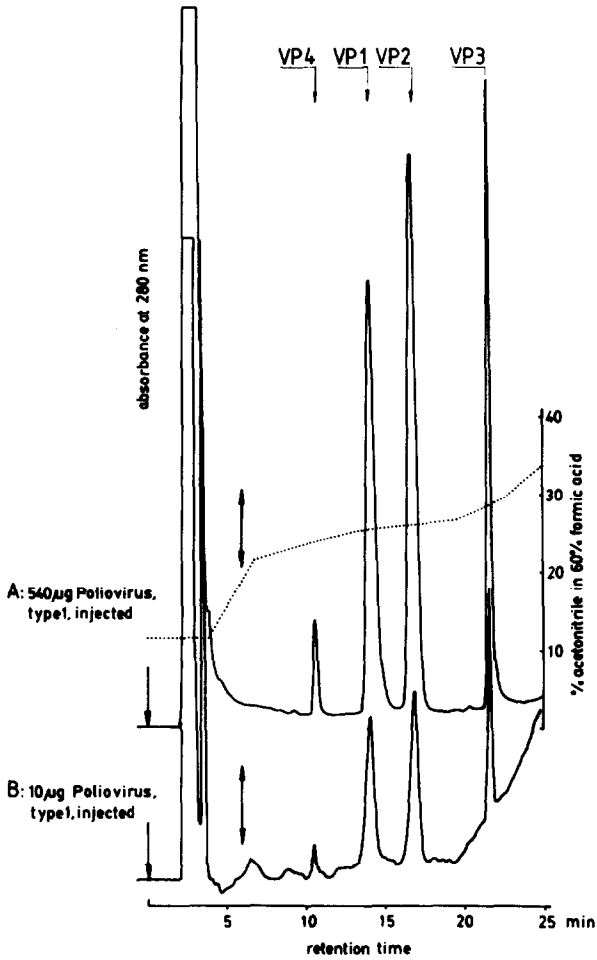
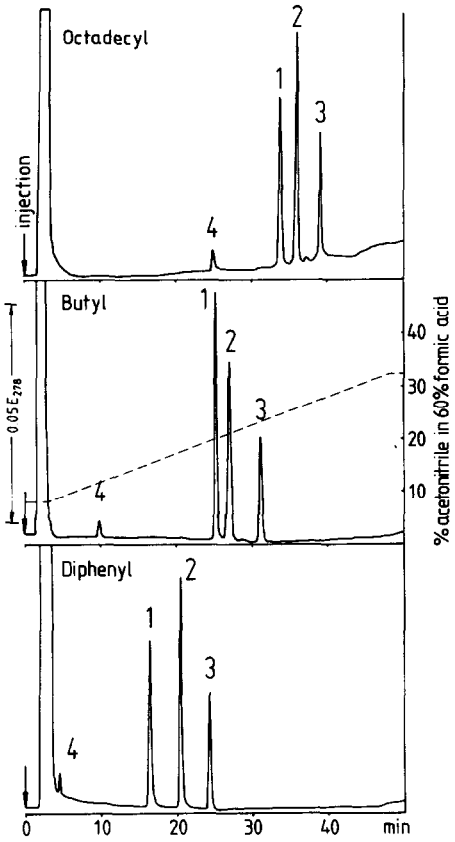


Fig. 1. Elution pattern of poliovirus polypeptides, separated by RP-HPLC on a micropreparative (A) and analytical (B) scale. Virus samples (poliovirus, Type 1, strain Mahoney) in 3 *M* CsCl were precipitated by 30% formic acid, redissolved in 50 µl of 70% formic acid and injected. Elution conditions: Baker widepore C_{18} column (250 × 4.6 mm), eluted by 60% formic acid with a gradient of acetonitrile, as indicated by dotted line. Flow-rate was 1 ml/min at 23°C. Absorbance range indicated by the arrows is 0.032 for A and 0.002 for B.

in the solvent system containing 60% formic acid, we investigated several of their physicochemical properties, such as size, hydrophobicity and charge (p*I*).

Effect on size

The purity of the isolated polypeptides and the fact that they are not altered in size was demonstrated by SDS-PAGE (Fig. 3). The acidic solvents with a high concentration of formic acid could induce hydrolytic cleavage of the polypeptide backbone, in particular, a cleavage of the acid-sensitive aspartyl–prolyl bond[42]. VP1, VP2 and VP4 do not contain this amino acid pair, but VP3 has three Asp-Pro pairs. To determine the sensitivity to acid, we stored poliovirus polypeptides (not separated)



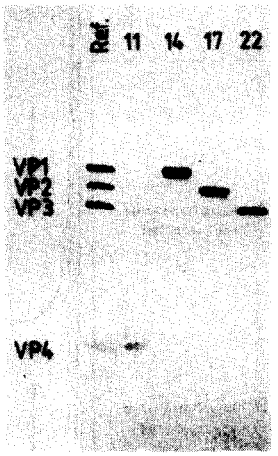


Fig. 2. Three samples of 55 μg poliovirus, Type 1, strain Mahoney, in 50 μl of 3 *M* CsCl were precipitated by 10% trichloroacetic acid, washed and redissolved in 6 *M* guanidine hydrochloride, containing 0.5% dithioerythritol. The samples were chromatographed under identical conditions with the exception of the column packings. Columns used: Baker Bond wide-pore octadecyl (C_{18}), butyl (C_4) and diphenyl. Elutions were performed with 60% formic acid as solvent A and 40% acetonitrile in 60% formic acid as solvent B. The gradient was linear, as indicated. The flow-rate was 1 ml/min at 25°C. Peaks 1–4 correspond to the poliovirus polypeptides VP1 to VP4.

Fig. 3. SDS-PAGE of poliovirus polypeptides (Type 1, strain Mahoney), separated by RP-HPLC. Aliquots of the peaks, eluted at retention times of 11, 14, 17 and 22 min, as shown in Fig. 1, were analyzed in lanes 2–5. SDS-dissociated poliovirus is in lane 1 as reference (Ref.).

for several hours in the eluent formic acid–20% acetonitrile at room temperature and at 4°C. The analysis shows (Fig. 4) that no cleavage occurred within 24 h for samples stored at 4°C. At room temperature, detectable cleavage was observed after 6–8 h.

Similar results were obtained for other proteins, separated by the mobile phase system used. Thermolysin and carbonic anhydrase, which contain two Asp-Pro bonds, are slightly cleaved after 6–8 h at room temperature, whereas trypsinogen, trypsin inhibitor and myoglobin, which contain no Asp-Pro pair, remain unaffected (not shown).

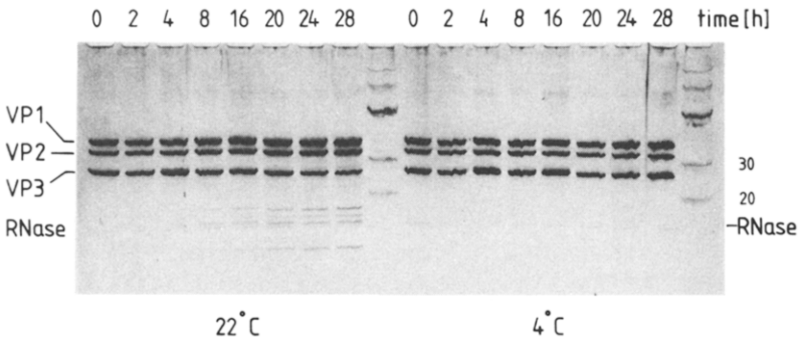


Fig. 4. Poliovirus, Type 1, strain Mahoney (30 μg), was dissociated in 8 *M* urea for 60 min at room temperature, RNA was digested with 5 μg RNase I for 30 min and the polypeptides were precipitated with trichloroacetic acid. The precipitate was dissolved in 800 μl of 60% formic acid, containing 20% acetonitrile. The solution of polypeptides was divided into 16 portions: 8 were stored at 4°C and 8 at room temperature (22°C). After the times indicated, samples were dried by vacuum evaporation and dissolved in sample buffer for SDS-PAGE, which was performed on an ultrathin gel layer of 12.5% polyacrylamide; 2.5% of each sample was applied to the gel. Molecular-weight-marker proteins were electrophoresed alongside (right, outer lane).

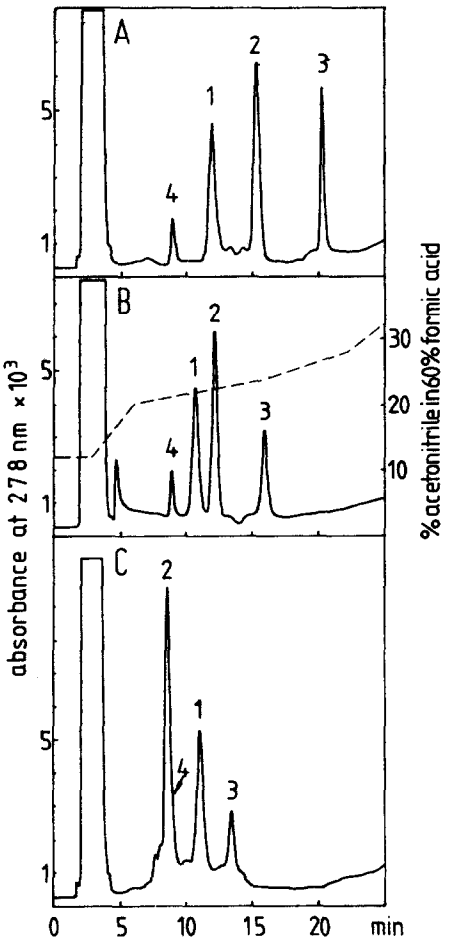
Effect on hydrophobicity

Modifications of proteins usually lead to an alteration of their hydrophobicity and, therefore, to shifts in their retention. On rechromatography of poliovirus polypeptides in the same solvent system the elution positions remain unchanged from those in the first separation. Thus, formic acid did not induce modifications. However, it is important for successful rechromatography to remove the formic acid without dehydrating the proteins. Therefore, water was repeatedly added to the collected fractions before they were dried completely.

The alkylation of thiol groups, which are present in low numbers in poliovirus polypeptides[36,43], causes a decrease in retention. This demonstrates that modification of only a few amino acids alters the elution behaviour. Of particular interest is the fact that vinylpyridine-modified virus polypeptides have a different elution order, because VP2 contains a greater number (8 Cys) of cysteines than VP1 (2 Cys) (VP3 contains 5 Cys)[36]. VP4 retains its elution position, because it does not contain cysteine (Fig. 5).

Effect on pI

The acidic mobile phase containing formic acid may catalyze deamidation of asparagine and, to a minor extent, of glutamine resulting in formation of pyroglutamyl residues. Also, formylaton reactions cannot be excluded completely. These types of reactions alter the relationship of acidic and basic groups within the proteins and would lead to shifts in their isoelectric points. Therefore, we studied some proteins, chromatographed in the formic acid system, by isoelectric focusing in polyacrylamide gels. The proteins which were tested by isoelectric focusing already showed multiple bands when dissolved in water. We have compared these patterns of bands, obtained from proteins which were not in contact with formic acid, with the ones from proteins which were chromatographed and stored in the formic acid–acetonitrile eluent for 1–6 h at room temperature.



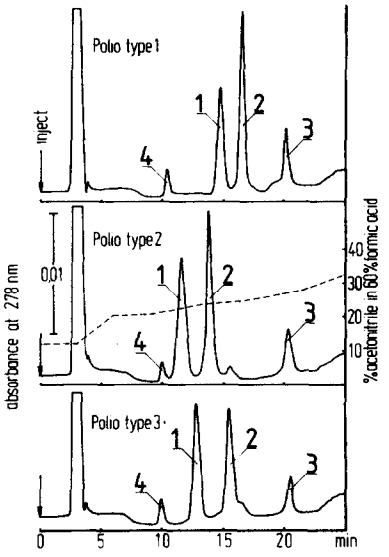


Fig. 5. Three samples, each of 15 μg poliovirus, Type 1, strain Mahoney, were precipitated with trichloroacetic acid, washed and redissolved in 6 *M* guanidine hydrochloride, containing 0.5% DTE, as in Fig. 3. One sample was chromatographed without derivatization as reference (A). To the second and third samples one equivalent (in relation to total thiol content) of iodoacetamide (B) or vinylpyridine (C) was added. After incubation at 37°C for 30 min, polypeptides were precipitated by ten-fold dilution in water, washed with ethanol, redissolved in 6 *M* guanidine hydrochloride and separated under identical conditions. Peaks 1–4 correspond to virus polypeptides VP1 to VP4. Conditions as in Fig. 1.

Fig. 6. Comparison of RP-HPLC separations of the structural polypeptides, originating in the three poliovirus types. Separation of Type 1, strain Mahoney, is shown in the upper chromatogram, of Type 2, strain MEF-1, in the middle and of Type 3, strain Saukett, in the lower chromatogram. About 30–40 μg virus in 50 μl of 3 *M* CsCl were precipitated with trichloroacetic acid, redissolved in 50 μl of 70% formic acid and injected for each chromatogram. Elution conditions as in Fig. 1.

With few exceptions, the patterns of the individual proteins were preserved after chromatography and storage in the eluent up to 6 h. Small differences in intensities and positions of a few bands were observed between proteins that had been chromatographed and not chromatographed, whereas the period of storage had no significant influence on the pattern of the bands.

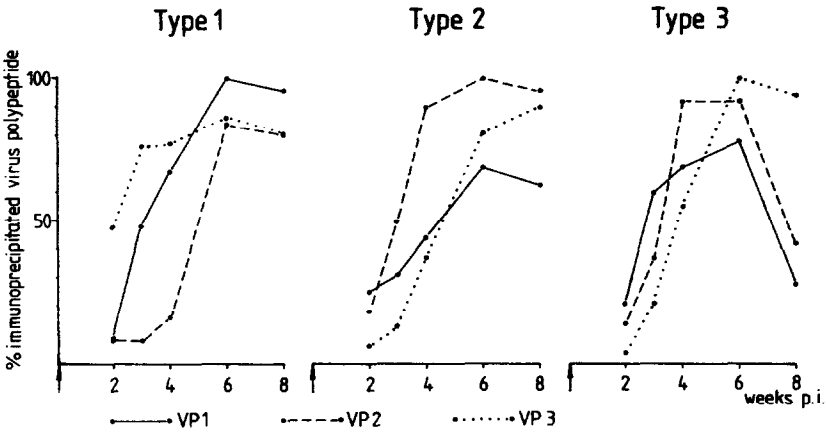


Fig. 7. Poliovirus polypeptides VP1, VP2 and VP3 of all three types were isolated by RP-HPLC, as shown in Fig. 6. Each polypeptide (200–250 μg) was mixed with complete Freund's adjuvant and injected subcutaneously into rabbits. Antisera were collected and tested by protein A-aided immunoprecipitation of [^{35}S]methionine-labelled polypeptides, obtained by dissociation (1% SDS, 100°C, 5 min) of poliovirus particles. The percentage of precipitated polypeptide was calculated from the radioactivity taking into consideration the number of methionine residues in the polypeptides[44].

Because chromatography and evaporation are rapid, the time of direct contact of proteins with formic acid is very short and degradation of the separated proteins can almost be excluded.

Effect on biological activity

Solubilization of water-insoluble proteins by urea, guanidine hydrochloride or detergents is usually accompanied by their denaturation. Undoubtedly, chromato-

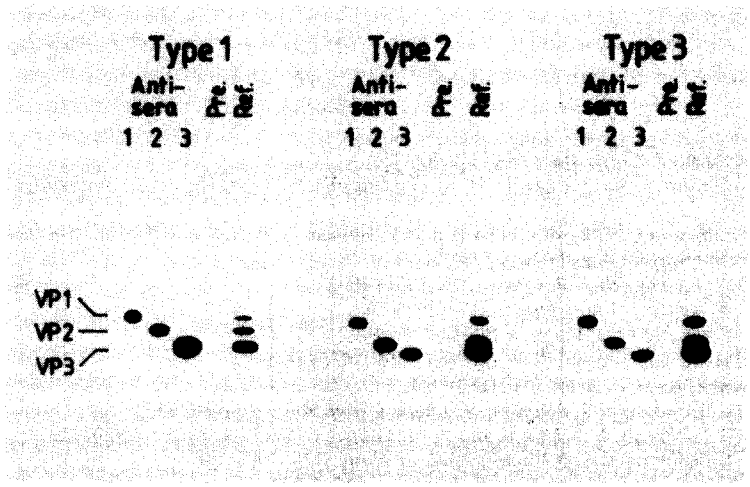


Fig. 8. The immunoprecipitated material (Fig. 7) was dissolved in electrophoresis sample buffer (pH = 6.7, 1% SDS) and subjected to SDS-PAGE and fluorographed[34]. Dissociated, but not immunoprecipitated poliovirus of the three types was used as reference (Ref.). Pre-immune sera gave no detectable polypeptide bands (Pre.).

graphy in formic acid also leads to denatured proteins, although some structural elements may be partially retained so that certain biological activities may be preserved. The biological activity of the separated poliovirus structural polypeptides may be expressed in terms of the antigenicity, which means, *e.g.*, the ability to induce antibodies, which react specifically with individual virus polypeptides as well as with intact virus particles.

We have separated and isolated the structural polypeptides of all three types of poliovirus on a micropreparative scale. Fig. 6 compares the chromatograms for analytical separations of the three types. Each type can be recognized by its characteristic chromatographic pattern.

The polypeptides VP1, VP2 and VP3 of each type were injected into rabbits. The antisera obtained were tested for their content of antibodies by immunoprecipitation (Fig. 7) and for their monospecificity by SDS-PAGE (Fig. 8) of SDS-dissociated radioactive poliovirus. About 6 weeks after immunization, all antisera showed the presence of monospecific antibodies; 70–100% of the homologous polypeptides were precipitated specifically by these antibodies (Fig. 7). Their monospecificity was a further indication of the high purity of polypeptides isolated by our RP-HPLC method.

Antisera directed against VP1, VP2, and VP3 were also bound to intact poliovirus particles and neutralized the virus at low, but clearly measurable antibody titres[4,5]. This demonstrates that the present method can be applied not only to chemical and biochemical but also to biological studies of proteins.

ACKNOWLEDGEMENTS

The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and by Bundesministerium für Jugend, Familie und Gesundheit, Bonn. The authors thank M. Hilbrig, U. Kuck and D. Weidemann for reliable technical assistance.

REFERENCES

1 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415.
2 W. S. Hancock, C. A. Bishop, R. L. Pretidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
3 J. Ozols, F. S. Heinemann and C. Gerard, C. Birr (Editor), *Methods in Peptide and Protein Sequence Analysis*, Elsevier/North-Holland, Amsterdam, 1980, p. 417.
4 J. J. L'Italien and R. A. Laursen, *J. Biol. Chem.*, 256 (1981) 8092.
5 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
6 K. J. Wilson, A. Honegger and G. J. Hughes, *Biochem. J.*, 199 (1981) 43.
7 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
8 W. C. Mahoney, *Biochim. Biophys. Acta*, 704 (1982) 284.
9 H. P. J. Bennett, C. A. Browne and S. Solomon, *J. Liquid Chromatogr.*, 3 (1980) 1353.
10 M. van der Rest, H. P. J. Bennett, S. Solomon and F. H. Glorieux, *Biochem. J.*, 191 (1980) 253.
11 C. A. Browne, H. P. J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
12 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.
13 J. J. L'Italien and J. E. Strickler, *Anal. Biochem.*, 127 (1982) 198.
14 E. C. Nice, M. W. Capp and M. J. O'Hare, *J. Chromatogr.*, 185 (1979) 413.
15 E. C. Nice, M. W. Capp, N. Cooke and M. J. O'Hare, *J. Chromatogr.*, 218 (1981) 569.
16 M. J. O'Hare, M. W. Capp, E. C. Nice, N. Cooke and B. G. Archer, *Anal. Biochem.*, 126 (1982) 17.

17 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.
18 G. E. Gerber and H. G. Khorana, *Methods Enzymol.*, 88 (1982) 56.
19 Y. Takagaki, G. E. Gerber, K. Nikei and H. G. Khorana, *J. Biol. Chem.*, 255 (1980) 1536.
20 G. J. Hughes, K. J. Winterhalter and K. J. Wilson, *FEBS Lett.*, 108 (1979) 81.
21 M. A. Phelan and K. A. Cohen, *J. Chromatogr.*, 266 (1983) 55.
22 B. R. Clark, J. A. Zaia, L. Balce-Directo and Y.-P. Ting, *J. Virol.*, 49 (1984) 279.
23 G. W. Welling, J. R. J. Nijmeijer, R. van der Zee, G. Groen, J. B. Wilterdink and S. Welling-Wester, *J. Chromatogr.*, 297 (1984) 101.
24 F. E. Regnier, *Methods Enzymol.*, 91 (1983) 137.
25 M. T. W. Hearn, *Methods Enzymol.*, 104 (1984) 190.
26 J. Heukeshoven and R. Dernick, *J. Chromatogr.*, 252 (1982) 241.
27 J. Heukeshoven and R. Dernick, *J. Virol. Meth.*, 6 (1983) 283.
28 J. Heukeshoven and R. Dernick, *Electrophoresis*, 6 (1985) 103.
29 B. J. Radola, *Electrophoresis*, 1 (1980) 43.
30 J. Heukeshoven and R. Dernick, in B. J. Radola (Editor), *Elektrophorese-Forum '83*, Institut für Lebensmitteltechnologie und Analytische Chemie, Technische Universität München, 1983, p. 92.
31 R. Drzeniek and P. Bilello, *J. Gen. Virol.*, 25 (1974) 125.
32 U. Yamaguchi-Koll, K. J. Wiegers and R. Drzeniek, *J. Gen. Virol.*, 26 (1975) 307.
33 J. L. Vaitukaitis, *Methods Enzymol.*, 73 (1981) 46.
34 K. J. Wiegers and R. Dernick, *J. Gen. Virol.*, 64 (1983) 777.
35 R. R. Rueckert, in H. Fraenkel-Conrat and R. R. Wagner (Editors), *Comprehensive Virology*, Vol. 6, Plenum, New York, 1976, p. 131.
36 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.
37 A. Hamann, C. Reichel, K. J. Wiegers and R. Drzeniek, *J. Gen. Virol.*, 38 (1978) 567.
38 H. B. Bull and K. Breese, *Arch. Biochem. Biophys.*, 161 (1974) 665.
39 K. A. Cohen, K. Schellenberg, K. Benedek, B. L. Karger, B. Grego and M. T. W. Hearn, *Anal. Biochem.*, 140 (1984) 223.
40 R. V. Lewis and D. De Wald, *J. Liquid Chromatogr.*, 5 (1982) 1367.
41 N. H. C. Cooke, B. G. Archer, M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
42 M. Landon, *Methods Enzymol.*, 47 (1977) 145.
43 J. Heukeshoven and R. Dernick, *Z. Naturforsch. C*, 36 (1981) 164.
44 H. Toyoda, M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura and A. Nomoto, *J. Mol. Biol.*, 174 (1984) 561.
45 R. Dernick, J. Heukeshoven and M. Hilbrig, *Virology*, 130 (1983) 243.