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ISOELECTRIC FOCUSING OF VIRAL POLYPEPTIDES IN UREA

A METHODOLOGICAL STUDY ON POLIOVIRUS

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SUMMARY

The applicability of isoelectric focusing in urea to the analysis of viral polypeptides is reported, using poliovirus as a model. Experimental techniques for the dissociation of virus particles, for isoelectric focusing in urea-containing polyacrylamide gels (rods, flat bed and slab gels) and for pH measurement and two-dimensional analysis are described and their results, as applied to poliovirus polypeptides, discussed. Special attention is given to problems of recovery of all of the proteins and the incidence of artifacts. The influence of reagents, dissociation conditions, focusing procedures and preparation and storage of virus material on the occurrence of charge modifications of the polypeptides has been investigated. Some recommendations are made for the application of the method to other viruses or particles.

INTRODUCTION

Isoelectric focusing was introduced in the early sixties[1] and, after the development of synthetic ampholytes[2], was soon established as a standard method for the separation and analysis of soluble proteins. Some reasons for the widespread use of this method are the convenience of determining isoelectric points and the high resolving power, especially in polyacrylamide gels. By use of isoelectric focusing, differences within individual charged residues in proteins can be detected[3–5], and additional information on the structure of protein can be obtained by specially designed experiments[4,6]. The applicability of the method has been improved by combination with gel electrophoresis in a two-dimensional separation. The bands obtained may be readily identified by determining their molecular weights[7,8].

Reliable procedures have been developed for isoelectric focusing of soluble proteins[9–11], but there have been few applications to particle-bound proteins, especially viral proteins[12–17]. Two-dimensional separation, with isoelectric focusing in the first dimension and sodium dodecyl sulphate (SDS)-gel electrophoresis in the second dimension, has been applied to chromatin[8], bacterial[5] or membrane[18] proteins,

ribosomes[19] and recently also to viral proteins[20,21]. Urea, partly with the addition of non-ionic detergents, is generally used as the dissociating agent. However, isoelectric focusing in urea still needs critical examination in relation to artifacts and to quantitative recovery before it can be considered a standard method like SDS-polyacrylamide-gel electrophoresis.

The present work investigates the applicability of isoelectric focusing and two-dimensional analysis to the polypeptides of viruses, using poliovirus as a model. Owing to the well-known polypeptide composition and simplicity of this virus, possible artifacts are more readily detected than with complex protein mixtures. Particular attention is given to the problem of quantitative recovery, and to steps in the experimental procedure which might lead to artificial changes in the charges of the proteins investigated. On the basis of experience with poliovirus, problems arising specifically from focusing in urea-containing gels are summarized and recommendations are made for a standard procedure for the application of the method to other viruses and particles. The virological aspects of the determination of isoelectric points and of the separation of poliovirus polypeptides have recently been briefly discussed[21].

MATERIALS AND METHODS

Materials

Ampholyte (Servalyte), acrylamide, Triton X-100 (pract.), SDS, bovine pancreatic ribonuclease A (RNase I, E.C. 3.1.4.22) were obtained from Serva (Heidelberg, G.F.R.). Ampholytes (Ampholine) from LKB (Bromma, Sweden) were also sometimes used. Acrylamide was recrystallized once from chloroform[22] before use, and urea was recrystallized, if necessary, from ethanol–water (1:1). In most experiments the ampholytes were purified before use with activated charcoal since, especially if stored for some months in a refrigerator, they gradually lost their capacity for sharp focusing in flat bed gels and gave more diffuse bands in rod gels. The ampholytes were diluted to give a 10% solution and then stirred for 1–2 h at room temperature with 0.1 g of charcoal (Norit A, Serva) per ml. After filtering through a membrane filter, the solution was frozen and stored. Nonidet NP 40 was obtained from Shell (Hamburg, G.F.R.), Coomassie brilliant blue from Gurr (Searle, High Wycombe, Great Britain). Other chemicals were obtained either from Serva or from Merck (Darmstadt, G.F.R.).

Virus. Poliovirus, Type I, strain Mahoney (unlabelled and ^{14}C-labelled) was grown in HeLa S_3 cells and purified as described elsewhere[23,24]. Two purification methods were employed, one using SDS (I), the other (II) including a precipitation with polyethylene glycol (methods 2 and 3, respectively, in ref. 23). Virus fractions were stored in 3 *M* CsCl at —20°. Before use, the material (0.2–1 ml) was concentrated, if necessary, to 4–8 mg of virus per ml by vacuum filtration and dialyzed against phosphate-buffered isotonic saline (PBS) for 2–4 h in a collodion finger at 0°. The optical density at 260 nm was measured for the determination of the virus concentration.

Dissociation

Dissociation was performed in polypropylene microlitre vials. In the standard procedure, 5 μl of virus in PBS were mixed with 45 μl of a 10 *M* solution of urea (re-

crystallized urea, stored at −20°) and 1 μl of RNase (5 mg/ml) and incubated for 1 h at 25°. If mercaptoethanol or dithiothreitol (DTT) was added, the vial was flushed with nitrogen before closing it.

Isoelectric focusing

Gels for isoelectric focusing contained 5% acrylamide (with 2.6% N,N'-methylenebis(acrylamide) or 15% N,N'-diallyltartardiamide[25]), 9 *M* urea and 2% ampholyte. The ampholyte comprised fractions of pH 5–7, 7–9 and 2–11 (1:1:0.5). Polymerization was initiated by ammonium persulphate (30 μl of a 10% solution for 10 ml of gel).

Isoelectric focusing in gel rods was performed in a simple apparatus for gel electrophoresis in which the gel tubes are cooled by the electrode solution (Fig. 1A). The gels were 2.5 or 5 mm in diameter and 8–10 cm in length. They were overlaid with 0.5–1 cm of 7 *M* urea containing 2% ampholytes and prefocused for 1–2 h at 500 V prior to application of the sample. Electrode solutions consisted of 0.1 *M* sodium hydroxide and 0.1 *M* phosphoric acid. After dissociation, 25 μl of the sample were carefully layered on top of the gel. The potential was 150 V overnight (or 5 h at 300 V), and an additional hour at 500 V.

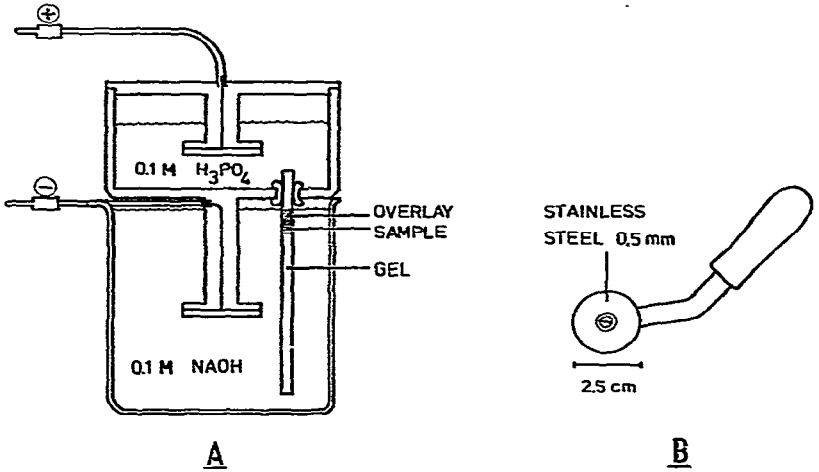

Fig. 1. (A) Apparatus used for isoelectric focusing in gel rods. (B) Wheel-blade for cutting slab or flat bed gels.

Slab gels (in closed chambers) were run in a slightly modified electrophoresis apparatus as described previously[26,27] with 1.3- or 2.5-mm thick chambers (14 × 16 cm). The chambers were supplied with spacers of glass, glued on one side, and strong plastic cramps, and required no additional sealing. Focusing conditions were the same as described for rods except that the potential was lower during prefocusing for 2.5-mm gels in order to reduce heating.

Flat bed gels (slabs on a glass support) with a thickness of 1 mm were prepared in the usual manner[9,28] and run in an apparatus with carbon electrodes lying directly on the gel[28,29]. The gel was kept at 25° by a thermostatted support. Dissociated virus samples were layered directly on to the prefocused gels near the anode. During the first hour the potential was 200 V, and for the following 2 h it was 1000 V.

Focusing under reducing conditions was achieved by layering 2-mercaptoethanol (diluted 1:10 in 8 *M* urea) or DTT dissolved in 8 *M* urea to give 50–100 m*M* in the gel on to the entire gel surface and allowing it to soak in before application of the samples. During focusing, nitrogen was blown through the apparatus. In order to prevent the gel from drying, the nitrogen was passed through a washing flask.

Two-dimensional analysis

In the first dimension, samples were focused in 2.5-mm gel rods or 1.3-mm slab gels. A 4–6 mm broad strip containing the separated polypeptides was cut out from the slab by means of a wheel-blade (Fig. 1B). Rods were equilibrated for 2 × 15 min in spacer-gel buffer[30] with 1% SDS, and for 15 min in buffer with 0.1% SDS. Strips from the slabs were equilibrated for half this time. If necessary, 1% 2-mercaptoethanol or 0.1 *M* DTT was included in the equilibration buffers. In some of the experiments the gel was heated to 100° for 2 min after the second equilibration step. For storage, the gels were frozen at −70° after the first or second equilibration step and stored at −20°.

In the second dimension, SDS-disc electrophoresis[30] was carried out in slab gels. The apparatus is the same as described for isoelectric focusing in slab gels. An 8-cm long separation gel (14% acrylamide) followed by a 1.5-cm spacer gel (3%) were cast and allowed to polymerize. The equilibrated gel rods or strips were introduced into the chamber and, after pouring a small amount of molten agarose (1% agarose in spacer-gel buffer with 0.1% SDS) below the rod or strip, they were pushed down on top of the spacer gel. Additional agarose was used to obtain a smooth surface. A plastic strip introduced previously at the side of the chamber was used to form a well for a reference sample. The chamber was mounted in the apparatus and, after underlaying the reference sample (virus dissociated for 2 min at 100° in spacer-gel buffer containing 1% SDS, 20% glycerol and 1% 2-mercaptoethanol), electrophoresis was performed at 100 V for 1 h and 250 V for an additional 2–4 h (Pulsed Power Supply, Ortec, Oak Ridge, Tenn., U.S.A.).

Staining and autoradiography

Gels from focusing or electrophoresis were stained with Coomassie blue solution (0.05% in 45% methanol–9% acetic acid containing 0.1% copper sulphate[10]) for a few hours or overnight, and destained in 40% ethanol–5% acetic acid. The staining solution was discarded after use. For staining gels containing mercaptoethanol or DTT, the copper sulphate was omitted. Slab gels, flat bed gels and 2.5-mm rods were dried in a vacuum on filter paper or Cellophane[30]. Another simple method for drying 5% gels has been reported recently[31].

For autoradiography, the dried gels containing 5000–10 000 cpm ^{14}C per sample were clamped together with X-ray film (Kodak Royal X-O mat) between glass plates and left in the dark for several days.

pH Measurement

For measuring the pH gradient in the gel (slab gels) after focusing, strips (0.5 × 2 cm) of gel were cut out close to the separated sample with a wheel-blade and eluted for 1–2 h in 200 µl of freshly prepared (or stored frozen) degassed urea solution (9 *M* recrystallized urea, 10–20 m*M* KCl for better conductivity[10]). The horizontal cuts

went some millimetres into the remaining gel for correlation of the pH measurements to the gel. The pH was determined at room temperature with a half-micro combined glass electrode (Radiometer, Copenhagen, Denmark).

If isoelectric points have to be measured repeatedly, use of protein markers is very convenient. Besides native proteins, such as bovine serum albumin, haemoglobin labelled with fluorescein isothiocyanate was useful because it revealed a large number of identifiable bands covering the range pH 8.4–6.5. The strongest bands are detected without staining, so that the progress of the focusing process can be observed. For coupling with fluorescein isothiocyanate, a haemoglobin solution (15 mg/ml in 0.1 *M* carbonate–bicarbonate buffer, pH 9.0) was mixed with fluorescein isothiocyanate (0.2 mg/mg protein, dissolved in acetone; final acetone concentration, 20%). After being allowed to react overnight at 4°, the preparation was purified on a short Sephadex G-25 column and freeze-dried.

The pI of the bands of this material, as well as that of other marker proteins, has to be determined under the given conditions since no data are available on the pI of proteins in 9 *M* urea.

RESULTS AND DISCUSSION

In a preliminary communication on the isoelectric focusing of poliovirus polypeptides[21], a correlation was made between bands found in electrofocusing and their molecular weights determined by SDS-gel electrophoresis. According to the established nomenclature, poliovirus polypeptides (VP) are identified by their molecular weight and characterized by the numbers 1–4 (ref. 32). The designations VP 1, VP 2, VP 3 and VP 4 will be used throughout this paper. The polypeptide VP 2 can occur in two strong bands in isoelectric focusing, the band with the lower pI being marked as VP 2_L. In order to avoid confusion, faint bands in the focusing patterns are not assigned.

Dissociation

In order to examine proteins of virus particles they have to be dissociated into their polypeptides and these must be kept in solution under appropriate conditions. The intermolecular forces holding the particulate structure together may be quite strong. This is demonstrated by the stability of some viruses, *e.g.*, poliovirus, to SDS at room temperature and neutrality[32]. Some of the routine dissociation methods are not suitable for isoelectric focusing studies since they use charged agents, such as guanidine hydrochloride, acetic acid and SDS. SDS has the additional drawback of binding tightly and denaturing the proteins irreversibly in most cases. Urea, which has also been used in isoelectric focusing and two-dimensional studies for the solubilization of ribosomes[19] and, in combination with non-ionic detergents, of membranes[18], has been employed in virology for a long time.

Standard conditions for the dissociation of poliovirus, Type I, strain Mahoney, are 9 *M* urea for 1 h at 25° in the presence of RNase[33,34]. Addition of RNase is essential; without it, part of the protein remains bound to the RNA and migrates to the anode during focusing (Fig. 2). The time, temperature and urea concentration needed for the dissociation are strongly dependent on the virus strain. Poliovirus, Type III, strain Saukett, for example, has to be dissociated at 37° for 60 min (ref. 34). In isoelectric focusing on flat bed gels, we found that the urea concentration in the

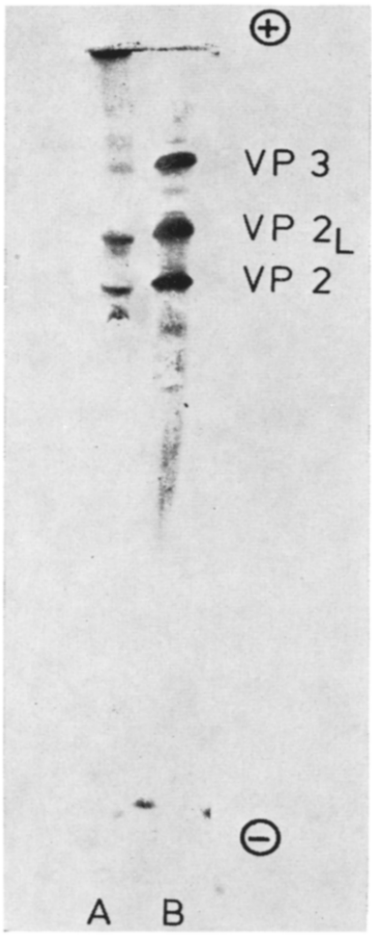

Fig. 2. Influence of RNase on the isoelectric focusing pattern of urea-dissociated poliovirus polypeptides on a stained flat bed gel. VP 1 has not focused in this experiment (streaked material). (A) No RNase added. (B) 0.1 mg/ml RNase present during dissociation.

gel has to be ≥ 7 *M*, otherwise VP 1 and VP 3 are not detected because they aggregate and do not penetrate into the gel (Fig. 3). This interpretation is in agreement with the analysis of urea-dissociated poliovirus by ultracentrifugation in sucrose gradients, where oligomers of VP 1 and VP 3 were observed[35].

When analyzing the pH dependence of the dissociation, it should be kept in mind that the pH of buffers is increased by urea[36]. PBS (pH 7.2) has a pH of 7.8 after dilution 1 : 10 with 10 *M* urea. In the absence of buffer, the pH value of urea solutions varies between 6 and 9: aged or sterilized urea solutions have a pH of 8–9. Under the conditions used (9 *M* urea, 1 h, 25°), we found no differences in the focused pattern of poliovirus, Type I, when the dissociation was performed between pH 5 and 8, which is in agreement with previous dissociation studies[34].

Dissociation of poliovirus by urea is influenced by the ionic strength[35]. This must also be considered in the focusing process itself. Assuming that the manufac-

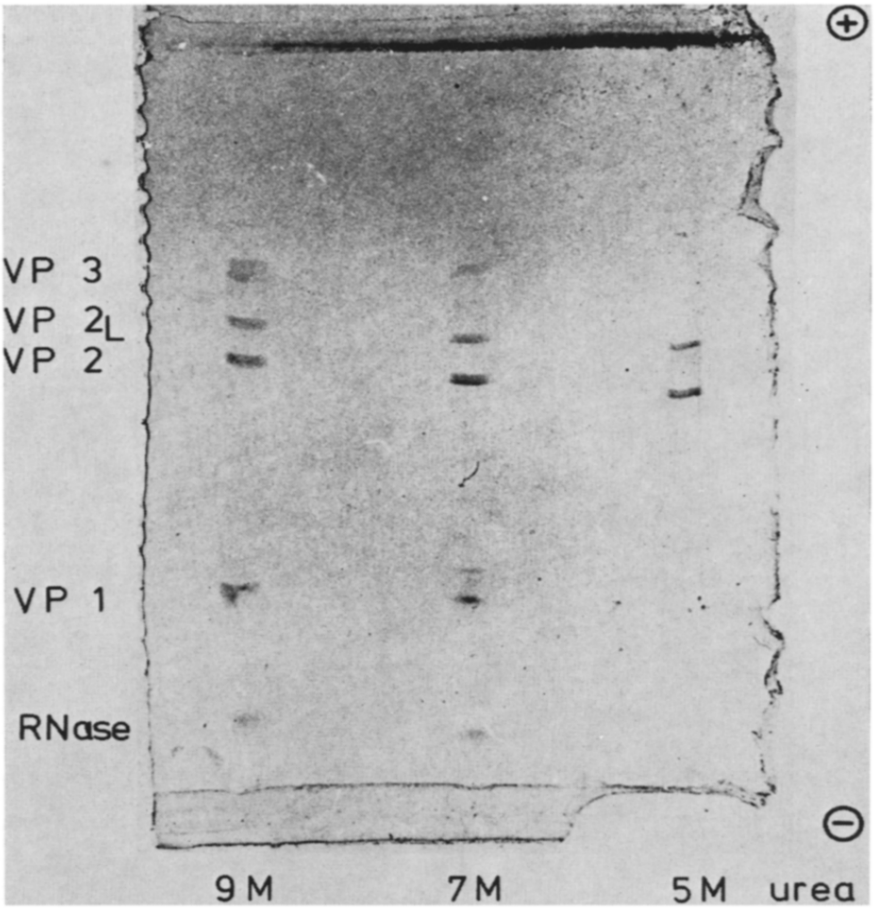

Fig. 3. Influence of the urea concentration in a flat bed gel. The gel contains an urea gradient at right angles to the direction of focusing. The urea concentration increases from right to left.

turers' data for the buffering capacity of ampholytes (*e.g.*, 0.8 mequiv./ml for Servalyte, pH 6–8) are equivalent to the ionic strength, a 2% ampholyte solution of the pH range used here would have an overall ionic strength of $\geqslant 0.05$ *M*. In gel electrophoresis of poliovirus in 10% polyacrylamide gels, in the presence of 8 *M* urea, part of the dissociated material (mostly VP 1) was found on top of the gel when 0.4 *M* Tris–citrate (pH 8.8) was used, but not if $\leqslant 0.1$ *M* Tris–citrate was used[37].

Isoelectric focusing

Fig. 4 shows the results of isoelectric focusing of poliovirus, Type I, in urea, in gel rods and slab and flat bed gels, using the techniques described in Materials and Methods. A high resolution is achieved and the polypeptides, including charge variants (secondary bands), are well separated despite small differences in their pI values. Gel rods and slab gels are found to be superior to flat bed gels both in resolution and recovery. The isoelectric point of the polypeptides can be determined easily and with good reproducibility, especially in slab and flat bed gels. However, the focusing in

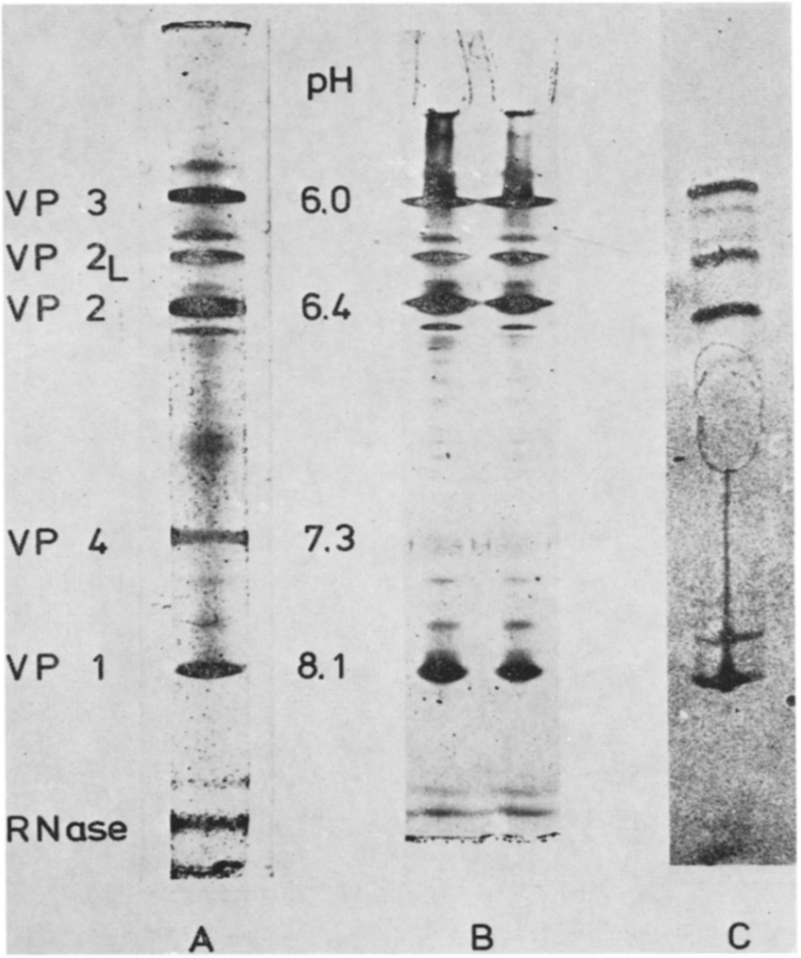

Fig. 4. Comparison of different techniques for isoelectric focusing of urea-dissociated poliovirus polypeptides. All of the gels contain 5% acrylamide, 9 *M* urea and 2% ampholyte. 5–10 μg of virus were applied. Stained gels. A = gel rod; B = slab gel; C = flat bed gel.

urea, in contrast to the results obtained with soluble proteins, has some restrictions with regard to quantitative recovery. Table I shows that the ratio of polypeptides found by scanning of stained gels or by counting the radioactivity of bands of labelled poliovirus after isoelectric focusing differs from the ratio reported for poliovirus particles, mainly for the polypeptides VP 1 and VP 4 Although the experimental procedure was essentially the same, a number of different factors can explain the poor recovery of these polypeptides. Some VP 4 seems to be lost from the large pore gel during staining and destaining owing to the small size of the polypeptide. In the case of VP 1, precipitated material on the surface can often be detected in flat bed gels with uncovered surfaces. Moreover, in this method, VP 1 often occurs as a smeared band (Fig. 4c) and sometimes does not focus at all (Figs. 2 and 9). These difficulties seem to be more pronounced when aged or untreated (with charcoal) ampholytes are used. It was also observed that virus polypeptides, especially VP 1 and VP 3, are partly

TABLE I

RATIOS OF POLIOVIRUS POLYPEPTIDES (% OF VP 2) AFTER ISOELECTRIC FOCUSING

(a) Ratio of polypeptides in poliovirus particles assuming equimolar amounts and molecular weights of 31000 (VP 1), 27000 (VP 2), 24500 (VP 3), 6000–8000 (VP 4)[38], on the basis VP 2 = 100%. (b) Ratio of poliovirus polypeptides after isoelectric focusing in gel rods. Experiments: 1–5 = scans of stained gels; 6 = relative radioactivity of cut bands of ^{3}H-labelled poliovirus (after staining). The amount of VP 2 + VP 2_L in the gel was set arbitrarily as 100%.

Experiment no.	*Ratio (%)*			
	VP 1	*VP 2 + VP 2_L*	*VP 3*	*VP 4*
(a)	115	100	91	20–30
(b) 1	31	100	94	4.5
2	66	100	71	—
3	89	100	94	6
4	109	100	95	2
5	20	100	91	8.5
6	40	100	68	6

adsorbed to the tube walls after dissociation[39]. When flat bed gels were covered with sheets of different materials during the run, complete adsorption of VP 1 and partial adsorption of VP 3 from the gel occurred with silicone rubber, polyethylene, silicone oil-treated polyethylene and Cellophane (dialysis membrane). In addition to the "stickiness" of these polypeptides, protein–protein interactions are also seen in urea solutions. Fig. 5 shows poliovirus polypeptides co-focused with bovine serum albumin. Part of the labelled virus material, obviously VP 1, focused with an altered pI between that of VP 1 and albumin. It should be mentioned that these adsorption phenomena occur not only when submicro amounts of highly labelled material are used but also, to a lesser degree, with higher amounts of unlabelled virus.

These observations show that urea is able to dissociate particles but will not abolish all of the protein–protein or protein–surface interactions. The strong adsorption to highly hydrophobic materials suggests that non-polar groups of the polypeptides, exposed by the dissociating and/or unfolding action of urea, may be involved in these phenomena.

The differences seen between the results of isoelectric focusing in gel rods and the less convenient flat bed gels seem to be related to the adsorption phenomena mentioned above, since an air–water interface is capable of interacting with the hydrophobic parts of proteins, often connected with a denaturing effect[40]. Attempts to overcome the smearing and stickiness by including amphipathic (aprotic) solvents, such as dimethyl sulphoxide, dimethylformamide and formamide, in flat bed gels either failed or resulted in poor focusing properties of the gel. It is not yet clear whether the poorer focusing properties of unpurified or aged ampholytes (see above) are due to the binding of ampholytes to virus polypeptides or with the phenomena mentioned in this section.

Surprisingly, addition of the non-polar detergents NP 40 or Triton X-100, as suggested for routine solubilization of hydrophobic membrane proteins[5,18], led to precipitation of VP 1 and VP 3 on or in the gel (Fig. 6). In the absence of other detergents or additives having a solubilizing effect, the adsorption phenomenon constitutes the main problem in the use of isoelectric focusing in urea, particularly for preparative purposes.

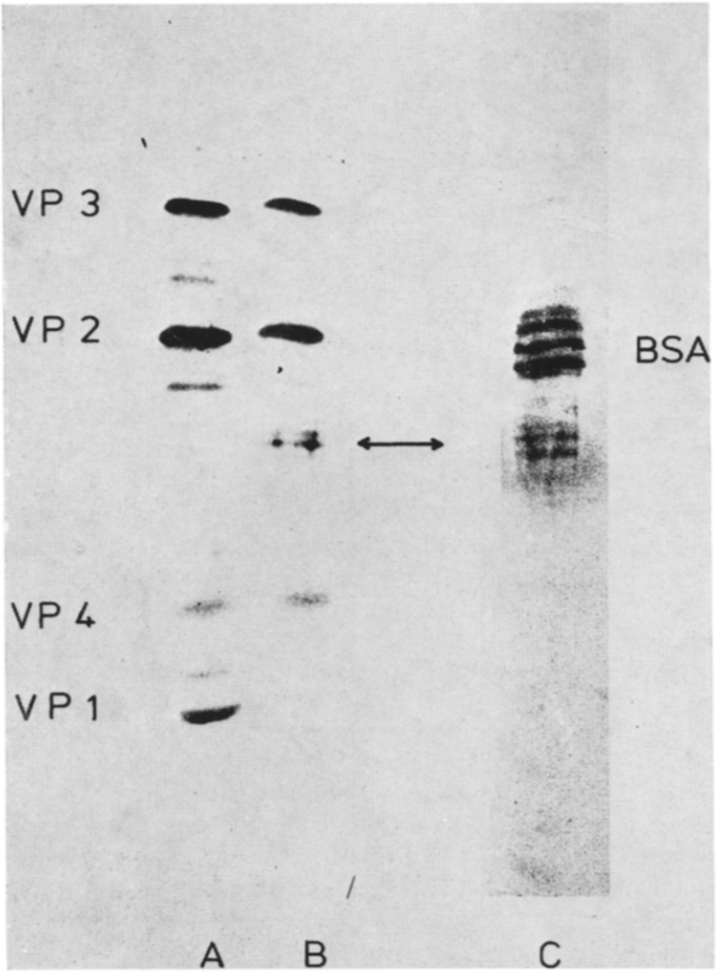

Fig. 5. Binding of poliovirus polypeptides to albumin during isoelectric focusing. Virus with and without addition of bovine serum albumin (BSA) was dissociated in urea and focused in a flat bed gel. (A) ^{14}C-labelled virus (5 μg), autoradiography. (B) Virus + 100 μg of albumin (1% solution in urea), autoradiography. (C) Same as B, but stained. The arrow marks the bands produced by interaction of VP 1 with albumin.

So far, problems connected with the quantitative aspects of recovery in urea-containing gels have been considered. However, the results of isoelectric focusing must also be scrutinized with respect to the qualitative findings, *i.e.*, the reliability of the isoelectric points found. Because of the high resolving power, a quite specific feature of isoelectric focusing is that very small differences in charge, *e.g.*, shifts produced in a single charged residue, are easily detected[3–5]. Most proteins investigated by focusing appear to be microheterogeneous, *i.e.*, they occur as isoelectric variants[41]. These may exist naturally, as in the case of glycoproteins with different sets of neuraminic acid residues[42], or they may develop postsynthetically, as in immunoglobulins[4]. Cleavage of amide groups, involvement of sulphydryl–disulphide transi-

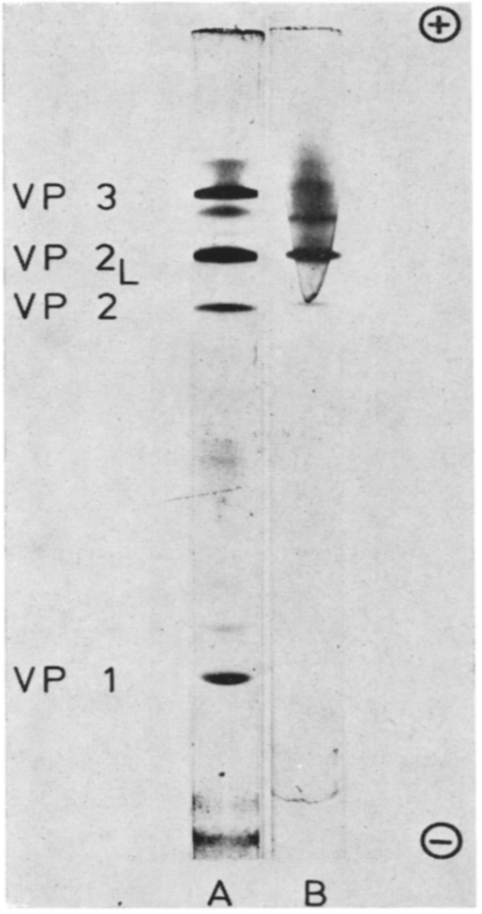

Fig. 6. Effect of the non-ionic detergent NP 40 on focusing of poliovirus polypeptides in gel rods. (A) Control. (B) 2% NP 40 present in the gel and dissociation mixture.

tions, phosphorylation or loss of terminal amino acids have been established as some of the causes of microheterogeneity[4,11,20,43–47]. The pattern seen after isoelectric focusing of poliovirus polypeptides shows, in most cases, more than one band for the virus polypeptides VP 1, VP 2 and VP 3. Fig. 7 shows the pattern of a two-dimensional separation allowing the correlation of the bands found in isoelectric focusing (upper part) to the four virus polypeptides detected by SDS-gel electrophoresis (left part). In order to determine whether the resulting isoelectric focusing pattern is due to natural or artificial charge differences within the respective polypeptides, we performed a series of experiments to pin-point the critical steps in the experimental procedure where charge changes could be produced. A direct verification of the protein sequences in different bands, *e.g.*, by peptide mapping, would be too laborious in most cases. However, differences in the pattern found in different experiments or after different treatments (see below) indicate the artificial charge changes are in fact responsible for the occurrence of more than four bands in isoelectric focusing of poliovirus polypeptides.

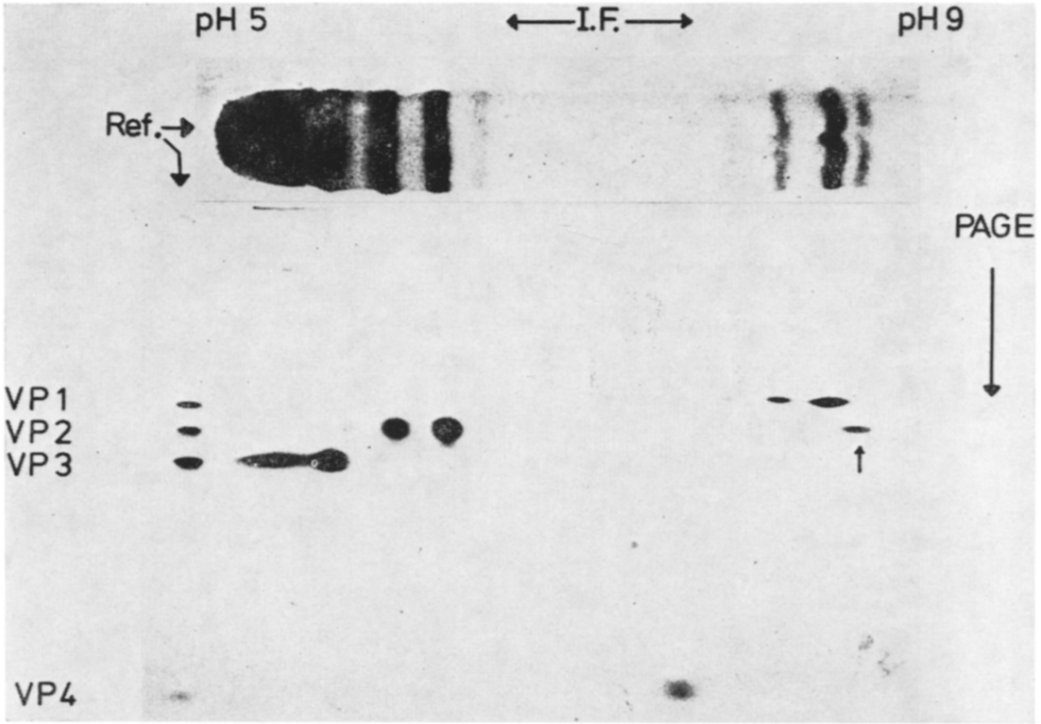

Fig. 7. Two-dimensional separation of poliovirus polypeptides in polyacrylamide gel. ^{14}C-labelled virus, autoradiography. First dimension (horizontal): isoelectric focusing (I.F.) in urea; top, reference gel. Second dimension (vertical): SDS-disc electrophoresis (PAGE); left side, reference sample of SDS-dissociated poliovirus. It is not known whether the spot labelled by an arrow represents a contamination of the preparation or a hitherto undescribed component of poliovirus particles. Denatured VP 3 has smeared on top of the I.F. gel (left side).

Possible reasons for artifacts are discussed in the following four sections. Not included is the possibility that charge variants of the polypeptides may be produced *in vivo, e.g.*, in connection with the proteolytic cleavage of precursor proteins or by postsynthetic modifications, as was found for the major capsid protein of Simian virus 40 (ref. 20).

Modification by the reagents used

It is known that a decomposition product of urea, isocyanate–cyanate, may react with basic groups of proteins leading to charge changes[48]. These decomposition products may be present or develop in considerable amounts also in urea solutions of reagent-grade material, as can be seen by measurement of the pH and conductivity. Although we found no effect of aging on the isoelectric focusing pattern of poliovirus polypeptides, it is advisable to use freshly prepared or frozen stored solutions of urea for the dissociation of virus and to recrystallize the urea once before use. This is less important for urea included in the gel since charged impurities are removed during a prerun. The use of high-quality acrylamide is also recommended. Acrylamide present as a 3% solution during dissociation (1 h, 25°) did not react with poliovirus polypeptides.

A point often mentioned is the effect of the polymerization catalysts ammonium persulphate and riboflavine. In contrast to most recommendations, we found persulphate to be more suitable than riboflavine. In riboflavine-polymerized gels additional artificial bands can occur compared to persulphate gels. Riboflavine, which is present during the dissociation, causes drastic charge changes at least in VP 2 (Fig. 8).

In method I, which yields the purest poliovirus material, SDS and non-ionic detergents are used during the purification procedure. In order to find out whether SDS, still bound to part of the polypeptides, causes the appearance of secondary bands, we added small amounts of SDS to the dissociation mixture (Fig. 9). The only

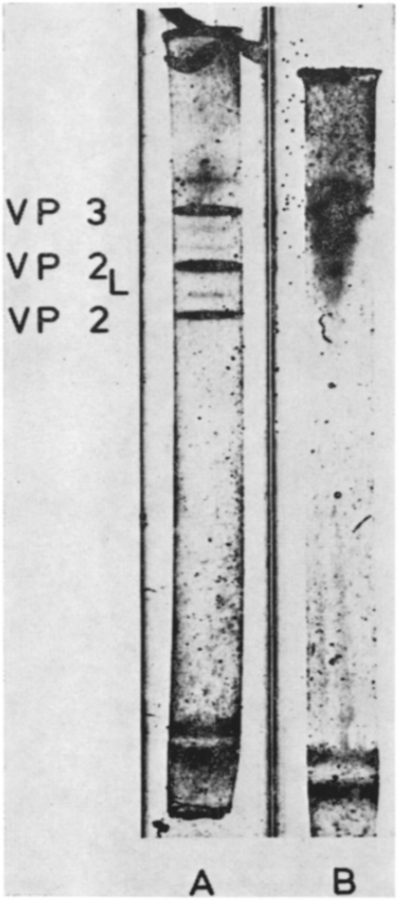

Fig. 8. Charge modification by riboflavin. (A) Control (standard dissociation). (B) 5 μg/ml riboflavin added to the dissociation mixture followed by exposure of the vial to light during dissociation.

Fig. 9. Effect of SDS on the focusing pattern of poliovirus polypeptides. Flat-bed gel focusing of the urea-dissociated virus. (A) 0.5% SDS added to the dissociation mixture. (B) Control (standard dissociation).

result was a loss of part of the material, but not a shift in the pI. Dialysis against non-ionic detergents also did not effect the band pattern.

Binding of metal ions might also affect the charge of the proteins. However, we found no influence of 2.5 m*M* EDTA, present during dissociation, on the focusing pattern.

It should be mentioned here that methods used for blocking sulphydryl groups are not always suitable for isoelectric focusing due to their insufficient specificity. A 50 m*M* solution of iodoacetamide present during dissociation produced a marked increase in the number of secondary bands (Fig. 10). Since the state of SH groups in poliovirus has no influence on their isoelectric point (see below), the observed behaviour must be due to non-specific side reactions with charged groups or to reactions producing charged groups on the polypeptide chain.

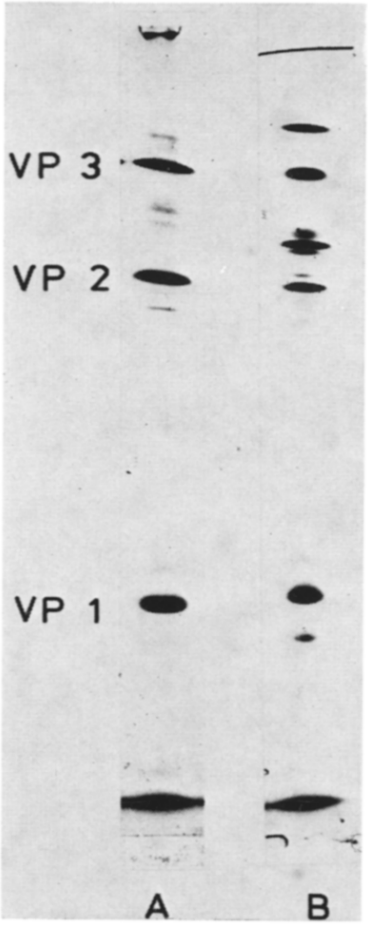

Fig. 10. Charge modifications by iodoacetamide. Urea-dissociated poliovirus focused in a slab gel. (A) Dissociation in 9 *M* urea, 0.1 mg/ml RNase and 0.1 *M* Tris–HCl, pH 8.3. (B) Same as A, but 50 m*M* iodoacetamide present during dissociation.

Artifacts produced by the focusing process

Only in a few cases were artifacts produced by the focusing process itself, provided some precautions were taken[49]. Relevant aspects are oxidation reactions, influences of pH and the site of application of the protein in the gradient and binding of ampholytes. Whereas the binding of ampholytes has rarely been demonstrated as the cause of distinct charge changes[11], the site of application is an important parameter, especially if narrow pH ranges and flat bed gels are used[50].

For poliovirus, the dissociated polypeptides have to be applied to flat bed gels at pH *ca.* 5–6. If applied at alkaline pH, the basic VP 1 runs into the cathode and is denatured, and VP 3 and VP 2 are lost after application just beside the anode. In gel rods or slab gels good results are obtained if the acid electrolyte is placed in the upper vessel and the protein mixture is protected from the acid pH by a sufficiently high overlay of ampholyte solution. The appropriate application site has to be found for all new applications and, if the recovery of all of the proteins is not convenient, a broader pH range has to be used. The problems connected with oxidation reactions are considered in the next section.

The verification of irreversible artifacts during the focusing procedure is obtained by refocusing. Upon inclusion of a gel strip of focused poliovirus polypeptides

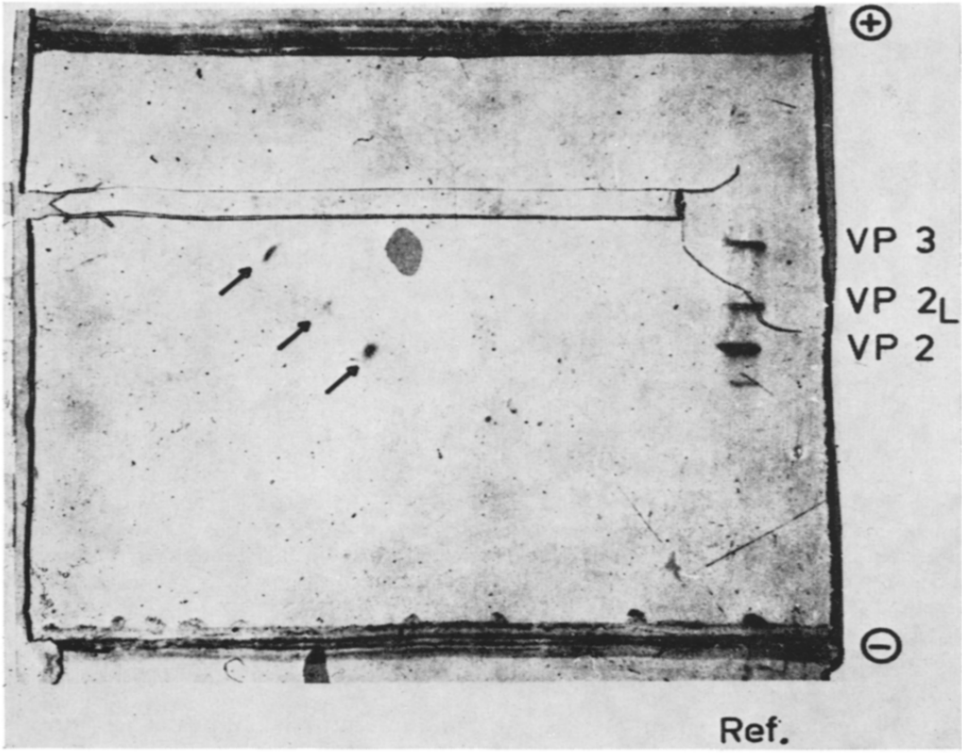

Fig. 11. Refocusing of poliovirus polypeptides. A strip containing the focused polypeptides of a previous run (flat bed gel) was cut out, included in a new prefocused gel and subjected to a second isoelectric focusing. Ref. = Reference sample of the second run. Arrows mark the spots of VP 2, VP 2_L and VP 3. VP 1 has not focused.

into a new gel perpendicular to the direction of focusing, and subsequent refocusing, the spots remained homogeneous and in the initial array (Fig. 11). This finding, and the different patterns seen in one gel when focusing different virus preparations in the same experiment (Fig. 12), indicate that the secondary bands are not produced by the focusing process but are dependent on virus preparation and storage.

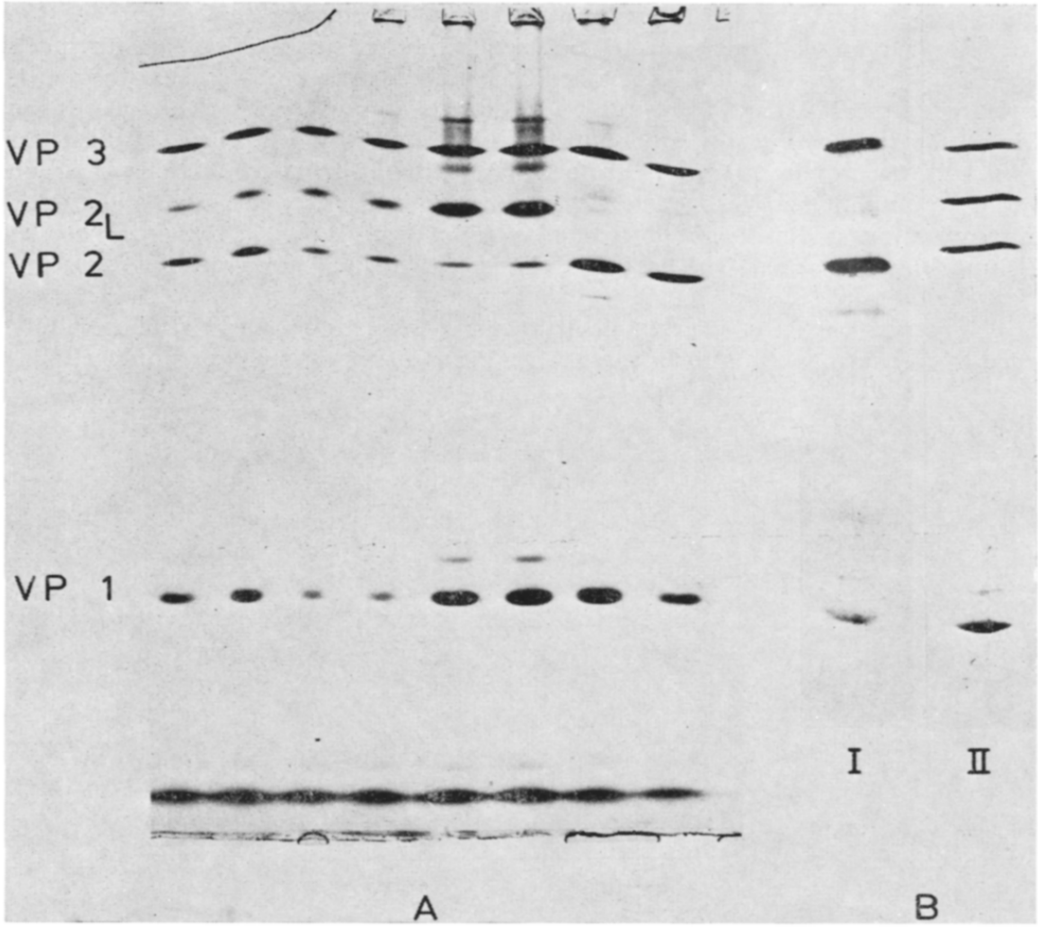

Fig. 12. Differences between various virus preparations. (A) Four different virus preparations were dissociated in urea and two samples of each focused together in a slab gel. The virus used in sample 1 + 2 and 5 + 6 (from the left) was prepared by method I, that in sample 3 + 4 (old) and 7 + 8 (fresh) by method II. (B) Appearance of the secondary band VP 2_L after storage in PBS. Same virus preparation: I = fresh; II = stored for 4 months at −20° (flat bed gel).

Influence of dissociation conditions

Dissociation of the virus is carried out using microlitre volumes. Therefore, it is relatively difficult to control the pH in only lightly buffered solutions, or to avoid oxidation. However, for poliovirus we could not find any difference in the focused pattern of virus material dissociated between pH 5 and 9. A change in the isoelectric point caused by oxidation-reduction can occur in at least two ways. First, by a direct

contribution of the proton of an SH group to the net charge of the protein: a prerequisite for this is that the pI of the polypeptide is not too far (<1–2 pH units) below the p*K* of the SH group in proteins (*ca.* 9.5)[51]. Secondly, by an indirect (steric) influence on the dissociation of charged groups of the polypeptide chains through formation of inter- or intra-molecular disulphide bonds. Additional charge changes are possible if oxidation of SH proceeds to cysteic acid, or if other drastic modifications are produced. In the case of poliovirus neither of these possibilities seems to occur. In contrast to SDS-gel electrophoresis, no differences were found between virus dissociated and focused in the presence of up to 5% mercaptoethanol or 0.1 *M* DTT.

Modifications occurring during preparation and storage of virus material

All of the evidence obtained so far indicates that the major isoelectric variants of poliovirus polypeptides are not produced by the reagents or the experimental conditions during dissociation and focusing. The following findings point to the incidence of modifications during preparation and storage of the virus material. First, there are significant differences between virus samples obtained by the two preparation methods. Method I always (six preparations) yielded virus material in which considerable, but different, amounts of VP 2 occurred as the isoelectric variant VP 2_L, whereas fresh preparations from method II (three preparations) were almost free from VP 2_L (Fig. 12A). Secondly, the pattern of an individual virus preparation may not remain stable: poliovirus, initially free from VP 2_L, shows considerable amounts of VP 2_L (Fig. 12B) after storage in PBS at −20°. It is interesting that the virus in 3 *M* CsCl at −20° is obviously completely stable. A 3-year-old preparation (method II) stored in CsCl was almost free from secondary bands of VP 2 (not shown).

Thus it can be concluded that the main isoelectric variants of poliovirus polypeptides are due to charge modifications occurring during virus preparation, depending on the method, and during storage of the dialyzed virus in PBS at −20°. It seems that the latter is the critical step. One of the differences between the purification methods is that in method I virus was stored in PBS at −20° before purification by centrifugation in CsCl gradients. We were not able to specify further the causes of the modification. Freezing and thawing (10 times) has no influence, nor could we find influences of the buffer (PBS or Tris–HCl), addition of oxidation-protecting agents (dithioglycol), dialysis time or incubation of the virus material after dialysis for 1 day at 25°. Considering the reported results, possible causes of charge modifications are deamidation, which has been shown to be responsible for the microheterogeneity of several proteins, or the action of a protease or other enzymes present in the virus preparation. More exact chemical analyses are required to clarify this point.

pH Measurement

Isoelectric focusing allows the determination of isoelectric points simply by measurement of the pH at the sites of the bands in the pH gradient. In the present case the usual technique[41] and the interpretation of data are somewhat complicated because of the presence of high urea concentrations. As already mentioned, the p*K* of charged groups is changed by urea, leading to a higher isoelectric point of a given protein[52]. The extent of the pH shift produced is dependent on the urea concentration[36,53]. This means that a change in urea concentration, *e.g.*, by evaporation during measurement of flat bed gels with a surface electrode, will give incorrect results. If

the pH is measured in eluted gel pieces, elution has to take place with an appropriate urea solution instead of distilled water. This urea solution must be stored frozen, recrystallized urea has to be used and the elution time should not exceed 1–2 h, otherwise the decomposition products of urea will affect the measured pH. An ideal technique would be the use of a small metal electrode, which can be pricked directly into the gel rod or flat bed gel, possibly through a thin protecting plastic sheet. An Ir–IrO_2 electrode was recently reported to have suitable properties[53,54].

Two-dimensional analysis

The two-dimensional analysis with isoelectric focusing in the first direction and SDS electrophoresis in the second direction allows quick identification of the bands separated by isoelectric focusing (Fig. 7). The principles and methodological aspects of SDS-gel electrophoretic separations are well established and need no further consideration here. The techniques for two-dimensional analysis have been described in great detail in recent publications[5,18].

Some recommendations for the application of the method

In order to facilitate the application of isoelectric focusing and two-dimensional analysis to viruses or other particles, the following recommendations resulting from our experiments are outlined.

(1) Select an appropriate dissociation procedure. 9 *M* urea and RNase for 1 h at 37° may be sufficient in most cases, but try to reduce the time and temperature later to avoid the risks of modification and adsorption. Addition of mercaptoethanol or DTT, *e.g.*, 0.1 *M* and EDTA (1–2 m*M*), is often mandatory. Do not use detergents without testing their action. The final protein concentration should be at least 0.1–0.5 mg/ml.

(2) Choice of the pH range and the application site of the sample. 5–15 μg of dissociated virus material are placed on prefocused gel rods containing ampholytes of broad pH range (2–11 or 3.5–10) and 9 *M* urea. Application at the anodic or cathodic end of the gel should be compared, and the pH determined in a reference gel. Use of flat bed gels facilitates these preliminary experiments. According to the results, the appropriate application site and, if desired, a narrower pH range can be selected. For narrow pH ranges, addition of 0.5% ampholytes having the broad pH range is useful in order to avoid contact of the protein with the electrode and electrode solutions.

(3) For final focusing use gel rods or closed slab-gel chambers. Slab gels are convenient for comparative purposes and pH measurement, but more of the expensive ampholytes are consumed and adsorption phenomena are more prominent. Slab gels and small (2.5-mm) rods can be dried in a vacuum after staining. Thick gels have to be stored in 7% acetic acid. Flat bed gels, although used excessively and successfully for isoelectric focusing of soluble proteins, should not be used for final studies in the applications mentioned here, since denaturation of sensitive proteins at the air–water interface may produce smearing and precipitations. Also, more difficulties have to be expected in uncovered flat bed gels owing to pH-gradient instability.

(4) For two-dimensional analysis, focusing is carried out in 2.5-mm gel rods or slab gels from which is small strip containing the separated sample is cut out. The gel rod or strip is equilibrated and SDS electrophoresis performed together with a

reference sample as described in Materials and Methods. The equilibration time may be shorter, as recommended in the literature, in order to avoid loss of small polypeptides. For autoradiography, thin gels (1–1.5 mm) dry more easily than thicker ones (2–2.5 mm). Gel rods of the first dimension can be introduced into such thin chambers by means of the smooth side of a well former, provided the edges of the chamber are rounded off by grinding.

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