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GEL CHROMATOGRAPHIC COMPARISON OF THE MOLECULAR WEIGHT DISTRIBUTIONS OF AMPHOLINE, SERVALYTE AND PHARMALYTE CARRIER AMPHOLYTES USED IN ISOELECTRIC FOCUSING

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SUMMARY

The molecular weight distributions of Ampholines, Servalytes and Pharmalytes have been studied by gel chromatography on Bio-Gel P-4. The results corroborate our previous conclusions with respect to the dependence of the molecular weight on the isoelectric point and on the carrier ampholyte system. Evidence for association of carrier ampholyte molecules was also obtained. The unexpected highly heterogeneous molecular weight distribution of Ampholines permits a better understanding of their composition.

INTRODUCTION

In a recent paper[1] we presented a comparison of the buffer capacities and specific conductivities of focused Ampholines, Servalytes and Pharmalytes. It was shown that the ratio of these two properties is related to the molecular weight of the carrier ampholytes. We found that (i) for the three systems studied the molecular weight generally increases with decreasing isoelectric point and (ii) the mean molecular weight increases in the order Servalytes $<$ Ampholines $<$ Pharmalytes.

In this paper we present results of a comparative gel chromatographic study, undertaken with the aim of verifying the former results independently. For this study a polyacrylamide gel (Bio-Gel P-4) was used, which appeared to have a higher resolution for the carrier ampholytes than dextran gels (Sephadex G-15 and G-25).

In determinations of molecular weight by gel chromatography it is essential to use as calibration standards compounds that are structurally related to the investigated substances. Firstly, if the separation mechanism is genuine gel permeation, elution volumes are governed by molecular size, which, in turn, is related to molecular weight only for a series of structurally related compounds. Secondly, if in addition to gel permeation other mechanisms are operative (*e.g.*, ion exchange, adsorption) the use of such a series is the only way of correcting for the effect of these mechanisms.

In the gel chromatographic molecular weight determinations of carrier ampholytes published thus far[2,3], polyethylene glycol standards were used for calibration.

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However, aliphatic compounds carrying the same functional groups should be used. For Ampholines polyaminopolycarboxylic acids (of the EDTA type) are the most appropriate (some amino acids, considered as the lowest molecular weight analogue in this series, can also be used). For Servalytes analogous compounds, with sulphonic and phosphonic acid groups, are suitable. For Pharmalytes polyamines carrying hydroxyl and amino acid groups would be ideal standard compounds.

Unfortunately, very few and rather low-molecular-weight compounds of these types are available. As peptides are the only more or less related series of compounds of which members with molecular weights up to a few thousand are available, we used them also for comparison. Further, the behaviour of the aminocarboxylic acids was compared with that of some low-molecular-weight aliphatic carboxylic acids and miscellaneous compounds.

EXPERIMENTAL

Bio-Gel P-4, fine grade (Bio-Rad Labs., Richmond, CA, U.S.A.), Sephadex G-25, fine grade, and Sephadex G-15 (Pharmacia, Uppsala, Sweden) were swollen in aqueous 0.2 *M* sodium chloride.

Columns (K16/70, Pharmacia) were filled with these gels by the slurry technique and packed to a bed height of 64.5 cm (bed volume 129 ml) at an elution rate of about 15 $ml \cdot h^{-1}$. The columns were equipped with a sample valve (LV4, Pharmacia), a flow adaptor (A16, Pharmacia), a peristaltic pump (Minipuls 2, Gilson, Villiers-le-Bel, France) and a UV monitor (Uvicord-S, LKB, Stockholm, Sweden).

As a routine, 15-μl aliquots of commercially supplied Ampholines (LKB), Servalytes (Serva, Heidelberg, G.F.R.) and Pharmalytes (Pharmacia), diluted with aqueous 0.2 *M* sodium chloride to 1 ml, were applied to the columns and eluted with aqueous 0.2 *M* sodium chloride at a flow-rate of about 15 $ml \cdot h^{-1}$. The flow-rate was determined gravimetrically. The UV absorbance was measured at 206 nm.

The following compounds were used for calibration or comparison:

Amino acids: glycine (Merck, Darmstadt, G.F.R.), proline (Serva, Heidelberg, G.F.R.), arginine (Hoffmann-La Roche, Basel, Switzerland).

Peptides: GlyGly (Merck), GlyGlyGly, AlaPhe, GlyGlyLeu, LeuLeuLeu, GlyPhePhe, PhePhePhe (all from Sigma, St. Louis, MO, U.S.A.), ProGlyLysAlaArg, ValValValValValVal (both from Serva), PhePhePhePhePhe (Sigma), thymus hormone, bradykinin potentiator C, fibrinopeptide A, α-endorphin, apamin, insulin A-chain (all from Serva).

Proteins: cytochrome *C*, bovine serum albumin (both from Sigma).

EDTA analogues: ethylenediaminediacetic acid (Aldrich Europe, Beerse, Belgium), nitrilotriacetic acid (Merck), ethylenediaminetetraacetic acid (Serva), ethylene glycol bis-(2-aminoethyl) tetraacetic acid (Fluka, Buchs, Switzerland), diethylenetriaminetetraacetic acid (Aldrich Europe).

Aliphatic acids: formic acid (Baker, Phillipsburg, NJ, U.S.A.), acetic acid, oxalic acid, succinic acid, tartaric acid, citric acid (all from Merck).

Miscellaneous: sodium azide, acetone, ethylenediamine (all from Merck), N,N-bis-(2-hydroxyethyl)glycine, N-α-(acetamido)-2-aminoethanesulphonic acid, N-2′-hydroxyethylpiperazine-2-ethanesulphonic acid (all from Serva).

Aliquots of 2–3 mg of these compounds, dissolved in 1 ml of aqueous 0.2 *M*

sodium chloride, were applied to the column and monitored as described above. Ethylenediamine was labelled with the ^{14}C compound (Radiochemical Centre, Amersham, Great Britain) to a specific activity of 16 nCi·μmol^{-1}. In that case 0.7-ml fractions of the eluate were collected and assayed for radioactivity by liquid scintillation counting.

RESULTS

In Fig. 1 the chromatograms of Ampholine, pH 6–8, on Sephadex G-15, Sephadex G-25 and Bio-Gel P-4 are given. As these results demonstrated that Bio-Gel P-4 gives the best separation of the Ampholine constituents, all further work was performed with this gel (see Discussion).

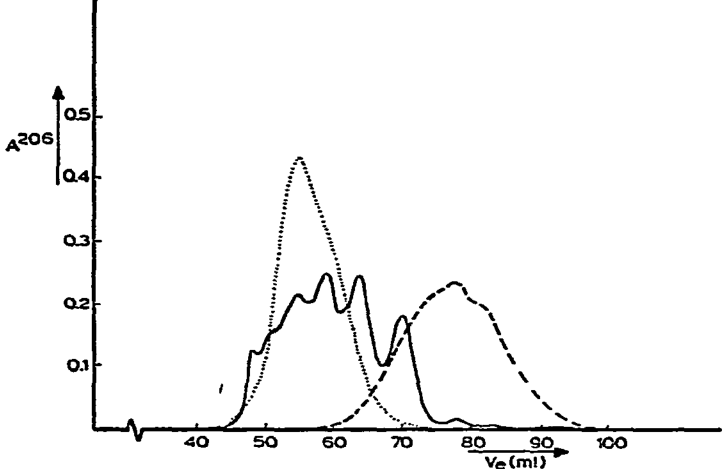

Fig. 1. Chromatograms of Ampholine, pH 6–8, on Sephadex G-15 (. . . . . .), Sephadex G-25 (———) and Bio-Gel P-4 (———). Bed height: 64.5 cm. Sample: 15 μl.

In Fig. 2 the elution volume of the standard compounds is plotted against the logarithm of their molecular weights.

Fig. 3 shows the influence of the sample concentration for Ampholine, pH 3.5–10.

In Fig. 4 the chromatograms for the neutral pH range of Pharmalyte at two concentrations and of Pharmalyte, pH 3–10, are shown.

In Fig. 5 the elution pattern of a new lot (see Discussion) of Pharmalyte, pH 5–8, at two concentrations is given.

In Figs. 6, 7 and 8 the elution patterns of an acidic, a neutral and a basic pH range of Ampholine, Servalyte and Pharmalyte, respectively, are given.

In Figs. 9 and 10 the elution patterns of the basic Ampholine ranges, pH 9–11 and pH 8–9.5 and those of the acidic Ampholine intervals, pH 2.5–4 and pH 4–6, respectively, are compared.

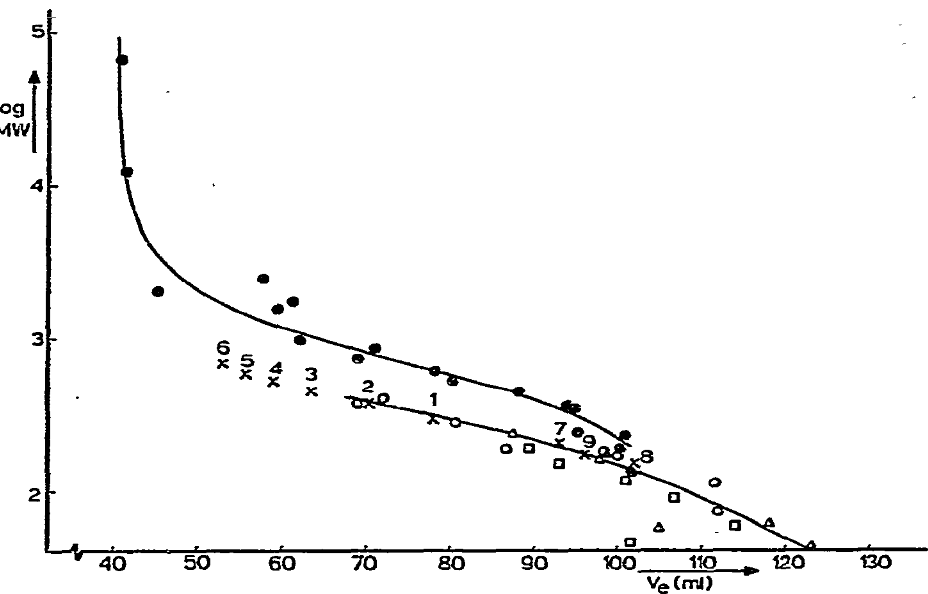

Fig. 2. Relationship between the logarithm of the molecular weights of standard compounds and their elution volumes, V_e, on Bio-Gel P-4. The standard compounds (for each category listed in order of increasing molecular weight) were as follows: *peptides and proteins* (●): GlyGly, GlyGlyGly, AlaPhe, GlyGlyLeu, LeuLeuLeu, GlyPhePhe, PhePhePhe, ProGlyLysAlaArg, ValValValValValVal, PhePhePhe PhePhe, thymus hormone, bradykinin potentiator C, fibrinopeptide A, α-endorphon, apamin, insulin A-chain, cytochrome C, bovine serum albumin; *EDTA analogues and amino acids* (○): glycine, proline, arginine, ethylenediaminediacetic acid, nitrilotriaceticacid, ethylenediaminetetraacetic acid, ethylene glycol bis-(2-aminoethyl)tetraacetic acid, diethylenetriamine pentaacetic acid; *aliphatic acids* (□): formic acid, acetic acid, oxalic acid, succinic acid, tartaric acid, citric acid; *miscellaneous* (△): sodium azide, acetone, ethylenediamine, N,N-bis-(2 hydroxyethyl)glycine, N-α-(acetamido)-2-aminoethane sulphonic acid, N-2'-hydroxyethylpiperazine-2-ethanesulphonic acid. The numbered points (×) refer to Ampholine constituents (see Discussion).

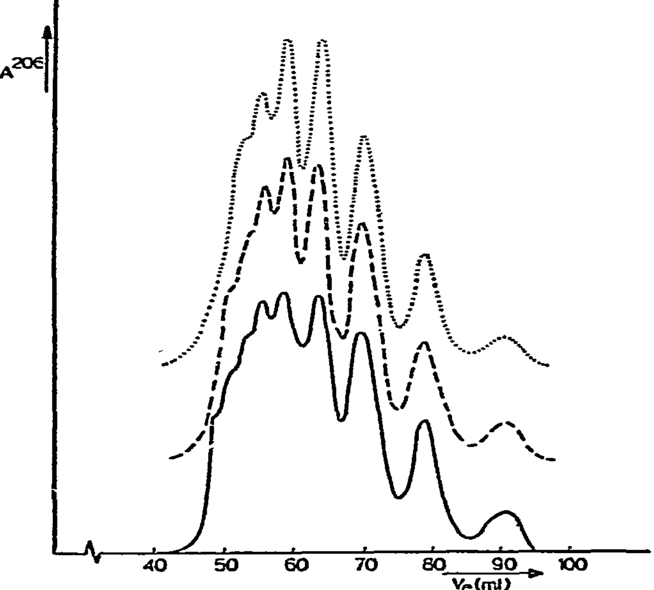

Fig. 3. Chromatograms of Ampholine, pH 3.5–10, at different concentrations, on Bio-Gel P-4. Samples: 15 μl (. . . . . .), 50 μl (-------) and 100 μl (———).

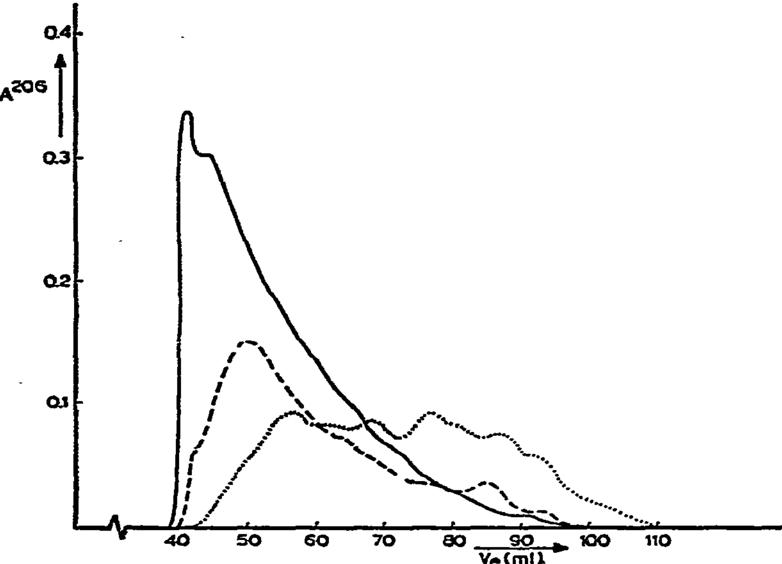

Fig. 4. Chromatograms of Pharmalytes on Bio-Gel P-4. Samples: 15 μl of Pharmalyte, pH 5–8 (———), 5 μl of Pharmalyte, pH 5–8 (------), and 10 μl of Pharmalyte, pH 3–10 (. . . . . .).

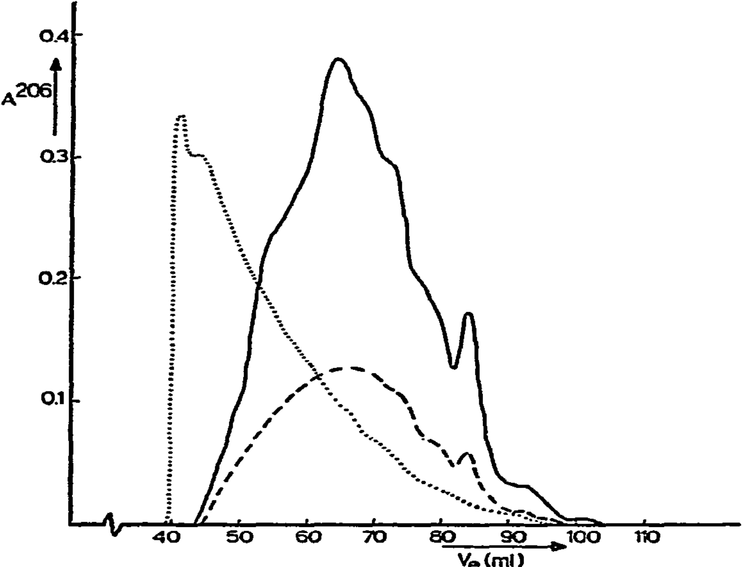

Fig. 5. Chromatograms of a new lot of Pharmalyte, pH 5–8 ,at different concentrations, on Bio-Gel P-4. Samples: 15 μl of Pharmalyte, pH 5–8 (———), and 5 μl of Pharmalyte, pH 5–8 (--------). For comparison, the chromatogram of an earlier lot of Pharmalyte, pH 5–8, from Fig. 4, is also given (. . . . . .).

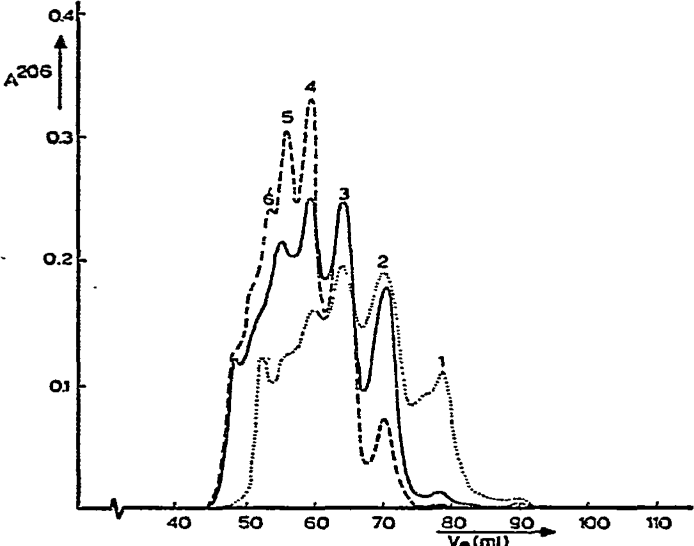

Fig. 6. Chromatograms of Ampholines on Bio-Gel P-4. Samples: 15 μl of Ampholine, pH 4–6 (--------), 15 μl of Ampholine, pH 6–8 (———), and 15 μl of Ampholine, pH 8–9.5 (......).

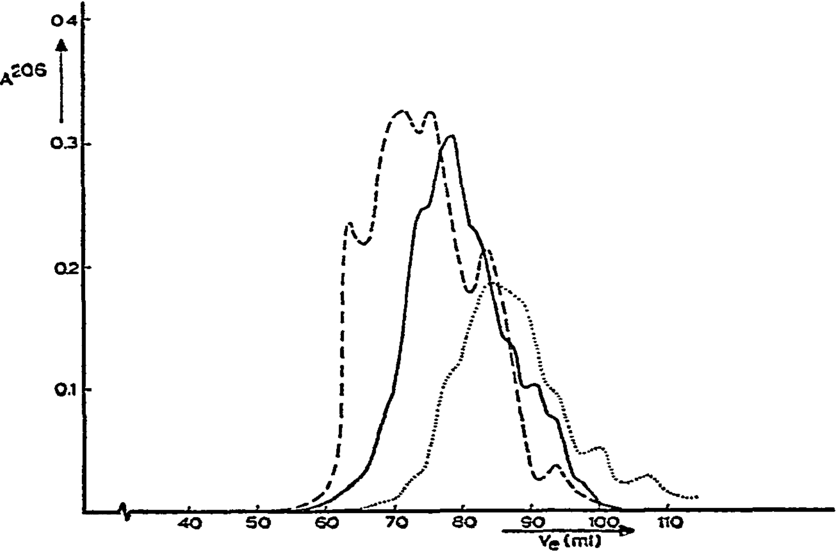

Fig. 7. Chromatograms of Servalytes on Bio-Gel P-4. Samples: 15 μl of Servalyte, pH 4–6 (--------), 15 μl of Servalyte, pH 6–8 (———), and 15 μl of Servalyte, pH 8–10 (......).

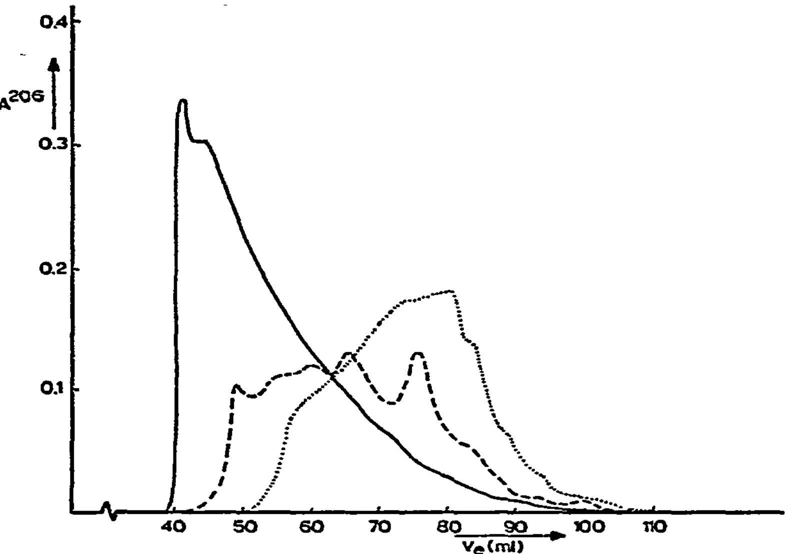

Fig. 8. Chromatograms of Pharmalytes on Bio-Gel P-4. Samples: 15 μl of Pharmalyte, pH 2.5–5 (--------), 15 μl of Pharmalyte, pH 5–8 (———), and 15 μl of Pharmalyte, pH 8–9.5 (......).

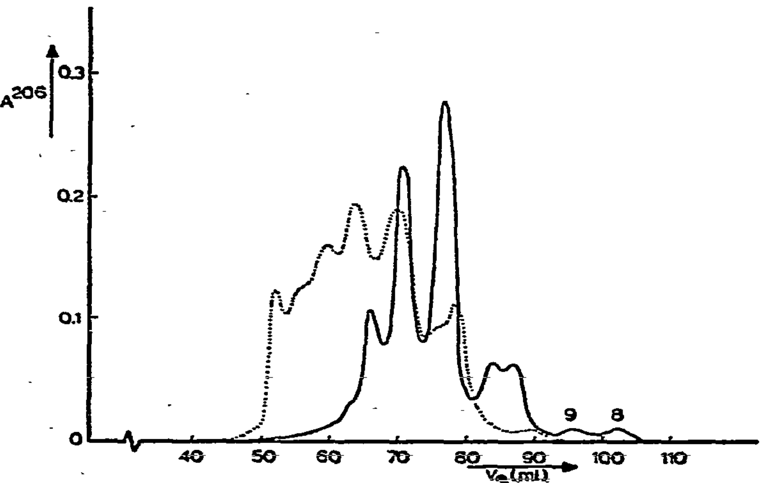

Fig. 9. Chromatograms of basic Ampholines on Bio-Gel P-4. Samples: 15 μl of Ampholine, pH 8–9.5 (......), and 15 μl of Ampholine, pH 9–11 (———).

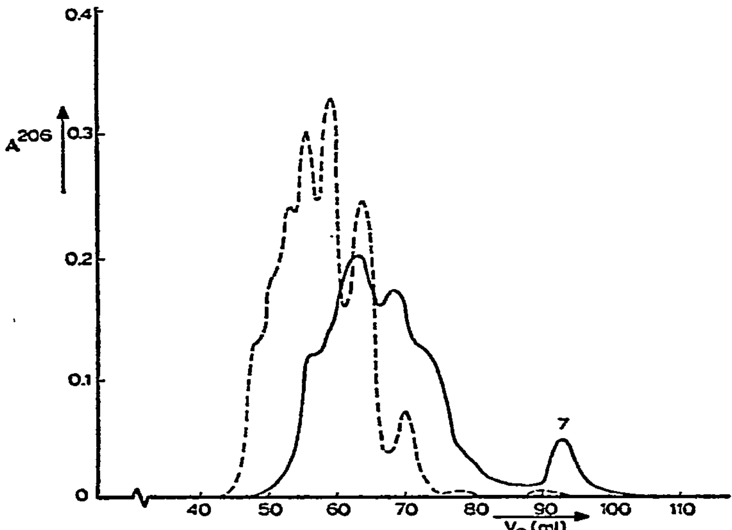

Fig. 10. Chromatograms of acidic Ampholines on Bio-Gel P-4. Samples: 15 μl of Ampholine, pH 4–6 (--------), and 15 μl of Ampholine, pH 2.5–4 (———).

DISCUSSION

Comparison of the gels

It can be seen in Fig. 1 that the resolution of Ampholine constituents by the gels increases in the order Sephadex G-15 < Sephadex G-25 < Bio-Gel P-4. For Sephadex G-25 and Bio-Gel P-4 this is partly due to a higher selectivity, *i.e.*, a smaller slope ($|d(\log M)/d(V_e)|$, where M = molecular weight and V_e = elution volume) of the calibration line, but mostly to a higher efficiency, *i.e.*, a smaller theoretical plate height with Bio-Gel P-4. The latter, determined from the elution peak of sodium azide (elution volume 123.0 ml), was 0.14 mm. In spite of several trials, we did not succeed in packing a Sephadex G-25 column with a better theoretical plate height than about 1 mm for the sodium azide elution peak*. A relatively smooth elution pattern on Sephadex G-25 was also obtained by other investigators[2,4] for ^{14}C-labelled Ampholine, pH 3.5–10. From the results in Fig. 1 we decided to perform all further work on Bio-Gel P-4.

Standardization of the gel

Fig. 2 clearly shows that peptides are retarded on Bio-Gel P-4 relative to com-

* The superior efficiency of Bio-Gel P-4 is due to its smaller maximum bead diameter and its narrower bead diameter distribution. For the fine grade of Bio-Gel P-4 used a hydrated bead diameter range of 37–75 μm is given by the manufacturer. The grades of Sephadex G-15 and G-25 used have, according to the manufacturer, dry bead diameter ranges of 40–120 and 20–80 μm, respectively. On the basis of the water regain[5] on swelling (1.5 and 2.5 $ml \cdot g^{-1}$, respectively), the density[5] of the swollen gel (1.19 and 1.13 $g \cdot ml^{-1}$, respectively) and a supposed density of the dry gels equal to that of sucrose (1.6 $g \cdot ml^{-1}$), this corresponds to hydrated bead diameter ranges of 64–192 and 34–136 μm, respectively.

pounds of the same molecular weight without a peptide bond. The content of phenylalanine residues appears not to be a significant factor in the retention of the peptides. We ascribe this specific retardation to interaction of the peptide bond with the carbamyl groups of the polyacrylamide, presumably by hydrogen bonding.

With the exception of the points for acetone and formic acid, those of all the other standard compounds fall approximately on the same smooth line. However, this line does not represent the true gel permeation behaviour. As the bed volume of our column is 129 ml and the specific packed bed volume of Bio-Gel P-4 is about 6 ml per gram of xerogel[5], the total volume of stationary and mobile phase must be about $5/6 \times 129 = 107.5$ ml, if the gel matrix is assumed to have a density of $1\ ml \cdot g^{-1}$. This volume is very close to the elution volume of acetone (105 ml), which therefore can be assumed to exhibit no interaction with the gel. This means, however, that all the other low-molecular-weight standard compounds (with the exception of formic acid) are retarded by some kind of interaction with the gel.

This agrees with the work of Streuli[6] on Bio-Gel P-2 and P-6 in 0.01 *M* sodium chloride solution. In that study acetone (and tetrahydrofuran, having the same elution volume) is also assumed to indicate the total volume. On this basis, Streuli found $K > 1$ on Bio-Gel P-2 for all of the carboxylic acids used in our study and also for citric acid on Bio-Gel P-6. Whereas such a strong retardation is not manifest in our work, as a result of the higher ionic strength used, some interaction must be assumed to explain our results.

The fact that peptides and EDTA analogues exhibit a significantly different behaviour on this gel precludes the determination of the molecular weight of material eluting in peaks in the elution patterns of Pharmalytes, which are known to contain at least one peptide bond[7]. Moreover, a general consequence of practical importance is that the separation of peptides and carrier ampholytes subsequent to isoelectric focusing is, at least on this gel at 0.2 *M* ionic strength, more difficult than could be supposed from the difference in molecular weight. On the other hand, the determination of the molecular weight of material eluting in peaks in the elution patterns of Ampholines (and Servalytes), using EDTA analogues and amino acids as standards, is possible. However, it is hampered by the considerable scatter of points around the calibration line (Fig. 2), and by the lack of EDTA analogues with molecular weights exceeding 400.

Association of ampholytes

In Fig. 3 the influence of the Ampholine concentration is shown. It can be seen that with increasing concentration definite shoulders gradually appear in the elution pattern at elution volumes of 51 and 48 ml. We ascribe this effect to increasing association of Ampholine constituents with increasing concentration. The Ampholine sample concentrations in these experiments were 0.6, 2 and 4% (w/v). In the chromatograms of the acidic and neutral Ampholine pH ranges (Fig. 6), where the Ampholine sample concentration was 0.6% (w/v), shoulders and even a small peak appear at the same elution volumes (51 and 48 ml). These can also be ascribed to Ampholine associates as the relative concentration of a particular Ampholine species is almost three times higher in these narrow pH ranges than in the broad pH range used in Fig. 3. As the usual ampholyte concentration in isoelectric focusing is, on average, 2% (w/v), it is possible that association of Ampholines is manifest in isoelectric focusing, as postulated by

Gianazza *et al.*[8]. However, it should be realized that the conditions in this gel chromatographic work and in isoelectric focusing are different: the ionic strength in our work is 0.2 *M* whereas in isoelectric focusing it is exceedingly small[9]; on the other hand, the high electric field strength prevailing in isoelectric focusing is absent, of course, in gel chromatography.

Neutral Pharmalytes also show considerable material eluting at small elution volumes, as can be seen from the chromatograms for the pH range 5–8 at concentrations of about 0.2 and 0.6% (w/v) in Fig. 4. Whereas this could be partly due to association also [in the chromatogram for the broad pH range 3–10 (Fig. 4), where the relative concentration of all species is small, no material is found at small elution volumes], Pharmacia pointed out to us[10] that Pharmalyte of pH 5–8 contains many ampholyte species of high molecular weight. As the company was changing the production procedure for this pH range material at the time this paper was being prepared and intends to sell the new product by the time it is published, we also chromatographed an aliquot of a new lot of Pharmalyte, pH 5–8. In Fig. 5 the elution pattern of this new lot, at concentrations of about 0.2 and 0.6% (w/v), is shown. As can be seen, the content of high-molecular-weight material is significantly reduced in this new product.

Relationship between molecular weight and isolectric point

The results in Figs. 6–8 substantiate one of our previous conclusions[1], *viz.*, that the molecular weight of carrier ampholytes generally increases with decreasing isoelectric point. This effect is most convincingly demonstrated for the Servalytes (Fig. 7), but also for the Ampholines (Fig. 6) there is a gradual shift towards smaller elution volumes with decreasing isoelectric point. The neutral Pharmalytes (Fig. 8) present an exception to this rule as their main fraction has a much smaller retention volume than the acidic and basic Pharmalytes. As argued in the preceding section, this could be partly due to association of neutral Pharmalytes.

Fig. 9 contains, for two basic Ampholine pH ranges, another confirmation of the general rule. In Fig. 10 the acidic Ampholine ranges, pH 4–6 and pH 2.5–4, are compared. It can be seen that, contrary to the general rule, the most acidic interval contains relatively more low-molecular-weight material. This confirms our earlier finding (*cf.*, Fig. 3 in ref. 1).

Comparison of the molecular weight distribution of Ampholines, Servalytes and Pharmalytes

Figs. 6–8 substantiate our earlier findings (*cf.*, Fig. 3 in ref. 1). The high molecular-weight boundaries of the molecular weight distributions increase in the order Servalytes $<$ Ampholines $\approx$ Pharmalytes for the acidic range, Servalytes $<$ Ampholines $<$ Pharmalytes for the neutral range and Servalytes $<$ Pharmalytes $<$ Ampholines for the basic range.

It is impossible to calculate mean molecular weights from the chromatograms. The ordinate in Figs. 3–10, absorbance at 206 nm, is proportional to the concentration of functional groups absorbing at 206 nm, *i.e.*, $>C=O$ for Ampholines and Pharmalytes and $>C=O$, $\geq P=O$ and $>S(=O)_2$ for Servalytes. Hence the sensitivity

of the measurement of the concentration of ampholyte species increases with increasing content of these functional groups, *i.e.*, in general, with decreasing elution volume. With Servalytes, however, nothing is known about the relative amounts of the absorbing groups in the ampholyte species. For the Pharmalytes we know neither the number nor the identity of the amino acids introduced. Even with Ampholines there remains some ambiguity as to the carboxylic acid content of ampholytes eluting in a particular peak (see the following section).

Assignment of the Ampholine peaks

The elution patterns of Ampholines (Figs. 3 and 6) show such well defined peaks that their identification can be attempted.

Of course, such an attempt is rendered difficult by the scattering of points around the calibration line (Fig. 2) and by the fact that we have no EDTA-like calibration compounds with molecular weights above 400, whereas most of the Ampholine peaks are eluted at smaller elution volumes than the highest EDTA analogue used. Therefore, the assignment proposed in this section must be regarded as highly speculative.

In a previous paper[1], the shift towards higher molecular weight with decreasing isoelectric point was explained as the result of a gradually increasing degree of substitution of amine hydrogens in a given polyamine with carboxylic acid residues. Hence, if the peaks in Fig. 6 are due to ampholyte molecules and if these molecules are separated on Bio-Gel P-4 according to their molecular weights, one might expect a shift of their elution volumes towards smaller values with decreasing isoelectric point. The peculiarity in Fig. 6 is, however, that almost all of the peaks appear at invariable elution volumes in the chromatograms for the acidic, neutral and basic pH range materials.

Therefore, we firstly ascertained that the peaks are due to genuine ampholyte species: although improbable in view of the high relative amount of material eluting in the peaks, they might be due to some contaminating non-ionizable compound, which, evidently, would be present in the three pH ranges and would elute at invariable elution volumes. For this purpose we chromatographed Ampholine, pH 6–8, on the cation exchanger SP-Sephadex at pH 4.9 and Ampholine, pH 4–6, on the anion exchanger DEAE-Sephadex at pH 8.2. Only on SP-Sephadex some UV-absorbing material eluted from the column in the break through volume, which upon re-chromatography on Bio-Gel P-4, however, did not reveal any peaks. In contrast, material eluting from the ion-exchange columns by the application of a salt gradient (0–1 *M* sodium chloride) did show the characteristic peaks upon re-chromatography on Bio-Gel P-4.

Secondly, we considered the possibility that the peaks in Fig. 6 are due to some specific interaction of Ampholines with the polyacrylamide gel, depending on the number of nitrogen atoms in the Ampholine constituents*, rather than on the

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* Ampholines are prepared[11] by the reaction of acrylic acid with a mixture of polyamines, the exact composition of which is unknown. The relevant patent[12] mentions polyamines with more than four nitrogen atoms; in a theoretical calculation of the maximum number of different ampholyte species obtainable in this synthesis, Vesterberg[13] assumes the presence of polyamines having between two and nine nitrogen atoms; in the more recent literature[14,15] pentaethylenehexamine (PEHA) is assumed to be one of the principal constituents of the mixture.

molecular weight. In that case, a particular peak would represent ampholytes derived from a particular polyamine, independent of the degree of substitution with propionic acid residues, *i.e.*, independent of the isoelectric point. In fact, Streuli[6] demonstrated that the amides acetamide, urea and biuret are adsorbed on Bio-Gel P-2 to an extent increasing with the number of nitrogen atoms when 0.01 *M* sodium chloride solution is used as the eluent.

Such a specific interaction with Bio-Gel P-4 was ruled out in our work, however. Firstly, the elution volumes of the calibration compounds are correlated with their molecular weights and not with the number of nitrogen atoms. Secondly, we re-chromatographed material eluting from Bio-Gel P-4 in the peaks marked 3, 4 and 5 in Fig. 6 on Sephadex G-25 and found that the elution order remained unchanged.

Thus, it must be concluded that the peaks in Fig. 6 are due to ampholyte molecules which are separated by molecular sieving. Assuming that the pricipal constituents of Ampholines are derived from PEHA by the introduction of propionic acid (PA) residues*, we assign the peaks n in Fig. 6 to PEHA·nPA ($1 \leqslant n \leqslant 6$). In Fig. 2 the molecular weights of these compounds are also plotted as a function of the elution volumes; it strongly suggests the correctness of this peak assignment.

However, an alternative assignment should be considered. In Fig. 11 the

```
-CH2-N-(CH2)x-N-CH2-          R2N-(CH2)x-N-(CH2)x-NR2
     |         |                          |
   (CH2)x    (CH2)x                     (CH2)x
     |         |                          |
    NR2       COOH                       NR2

where R=H or -(CH2)x-COOH     where R=H or -(CH2)x-NR2 or -(CH2)y-COOH
and x = 2 or 3                and x = 2,3 or 4 ; y = 1 or 2

        (a)                           (b)
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Fig. 11. General formula of Ampholines, according to the manufacturer[11] (a) and to Vesterberg[17] (b). The uncertainty with regard to the length of the carboxyl-connecting alkyl chain has been discussed by Righetti *et al.*[16]. As $y \neq 2$ is in contradiction with the stated[11] use of acrylic acid in the synthesis, we assume with these authors that only propionic acid residues ($y = 2$) are present in the normal-range Ampholines (p*I* 3.5–10).

general formula of Ampholines, as given by the manufacturer[11], is depicted. This formula allows the presence of polypropylenepolyamine, *e.g.*, pentapropylenehexamine-(PPHA) containing constituents, if $x = 3$ for *all* the nitrogen-connecting alkyl chains. Similarly, according to the general formula given by Vesterberg[17] (see also Fig. 11), even ampholytes derived from polybutylenepolyamines, *e.g.*, pentabutylenehexamine (PBHA), could possibly be present. The molecular weights of PEHA·nPA and PPHA·$(n-1)$PA and of PPHA·$(n-1)$PA and PBHA·$(n-2)$PA differ by only 2, corresponding to a difference in elution volumes of about 0.2 ml. Such a small difference could easily have remained undetected in our experiments. In fact, as PEHA·nPA, PPHA·$(n-1)$PA and PBHA·$(n-2)$PA are expected to have widely different p*I* values, this would provide an explanation for the presence of peaks (with $n \geqslant 3$) at virtually the same elution volume in the elution patterns for different pH ranges. In spite of this, we do not believe in the presence of appreciable amounts of

* See footnote on p. 311.

these compounds. Firstly, we presume that the statement $x = 2, 3$ or 4 in conjunction with the above-mentioned formula is meant to cover the presence of ampholytes in which some, but not all, x values are different from 2. Secondly, if substantial amounts of ampholytes with $x = 3$ or 4 throughout were present, one would expect Ampholines to show adsorption on hydrophobic materials. We did not find, however, any adsorption in hydrophobic interaction chromatography on octyl-Sepharose under conditions (1 *M* sodium chloride) where the tripeptides GlyGlyVal, GlyGlyLeu and LeuLeuLeu, containing isopropyl and isobutyl chains, are significantly retarded[18].

Therefore, another explanation must be found for the coincidence of most elution peaks in the chromatograms for different pH ranges. Inasmuch as relatively small peaks or shoulders are concerned (*e.g.*, peaks 1 for the neutral and acidic Ampholines and shoulder 5 for the basic Ampholines) they could be due to PEHA ampholytes with an isoelectric point outside the stated pH range. As Ampholines are fractionated, subsequent to their synthesis, by isoelectric focusing at high concentration, some species could remain in the "wrong" pH range as a result of association with the main constituents. Upon dilution, as in our experiments, such complexes dissociate and the constituents would appear individually in the elution pattern. This explanation cannot apply, of course, to the prominent peaks in Fig. 6, as substantial amounts of such "wrong" ampholytes would adversely affect the pH gradient obtained in isoelectric focusing.

We think that for these prominent peaks the explanation is provided by the presence of ampholytes derived from the different PEHA isomers. It has been shown by Vesterberg[19] that upon the substitution of amine hydrogens of a polyamine with alkyl carboxylic acid groups the pK values of the amine groups are virtually unchanged. Thus, the pK values of the amine groups in the original polyamine can be used in a rough estimation of the pI values of ampholytes derived therefrom provided that the degree of substitution is low enough that the pK value of the carboxylic acid plays no role in determining the isoelectric point. In Table I the four steric isomers of PEHA are schematically depicted; also indicated are the number of primary, secondary and tertiary amine groups.

It can be seen that with increasing branching the number of primary amine

TABLE I

THE FOUR STERIC ISOMERS OF PENTAETHYLENEHEXAMINE (PEHA)

PEHA isomer	*Number of amine groups*		
	Primary	*Secondary*	*Tertiary*
α: o—o—o—o—o—o	2	4	0

β: o—o—o—o—o
  |
  o
	3	2	1

γ: o—o—o—o—o
    |
    o
	3	2	1

σ: o—o—o—o
  | |
  o o
	4	0	2

groups (having relatively high p*K* values) increases, whereas the number of secondary amine groups (with relatively low p*K* values) sharply decreases. Hence, the introduction of, say, two PA groups into α-PEHA results in an ampholyte, the p*I* of which is determined by the p*K* values of one primary and one secondary amine group, whereas the same substitution into β-, γ- and δ-PEHA gives ampholytes with higher p*I* values, as they are governed by the p*K* values of two primary amine groups. Analogously, upon the introduction of three PA groups into PEHA, ampholytes will result with p*I* values, governed by two secondary (α-PEHA), one secondary and one primary (β- and δ-PEHA) and two primary (δ-PEHA) amine groups and therefore the p*I* value of PEHA·3PA isomers increases in the order $\alpha < \beta, \gamma < \delta$. Unfortunately, the magnitude of the p*I* difference between isomeric PEHA ampholytes cannot be given with certainty, as only the p*K* values of δ-PEHA are known[20]. We may assume, however, that the p*K* values of α-PEHA are equal to those of the linear tetraethylenepentamine isomer[20]. In Table II these values are given, together with the calculated p*I* values.

On the basis of these data, α-PEHA·3PA is expected to be present in Ampholine, pH 6–8, and δ-PEHA·3PA in Ampholine, pH 8–9.5, while both PEHA·3PA ampholytes of which, of course, many isomers exist, depending on the position of the PA residues, would appear at the same elution volume (*viz.*, that of peak 3 in Fig. 6). According to the above reasoning, peak 2 of the acidic Ampholine pH range cannot be PEHA·2PA. Nor can this peak be due to an ampholyted erived from a cyclic polyamine with only one primary amine group (Bergstedt and Widmark[21] demonstrated the presence of such piperazine derivatives in commercial PEHA). One would then expect a slight displacement (of about 2 ml) of the corresponding elution peaks, as these piperazine derivatives are not isomeric with PEHA, but differ by 26 molecular weight units from it. Such a displacement has not been observed, however. [Retardation on the gel of such piperazine derivatives relative to non-heterocyclic compounds, so as to give identical elution volumes, is improbable, as the piperazine derivative HEPES behaves as expected on the basis of its molecular weight (see Fig. 2)].

The well defined peaks in the elution patterns in Figs. 3 and 6 indicate that some constituents (*viz.*, if the above assignment is correct, PEHA isomers) of the polyamine mixture used in the synthesis of Ampholines either are predominant or react more efficiently with acrylic acid. This means that less different ampholyte species are present in normal-range Ampholines (pH 3.5–10) than could be assumed hitherto from the given general formulae (see Fig. 11)*.

In relation to the discussion of Vesterberg[17] on the extension of the pH range of Ampholines at both ends of the pH scale, *i.e.*, below pH 3.5 and above pH 10, it is of interest to reconsider the elution patterns in Figs. 9 and 10. As already argued previously[1], these comments can be taken as indicative of the hypothesis that the most acidic Ampholines are produced in different runs, *i.e.*, that they do not belong to the PEHA·*x*PA family. The same argument holds for the most basic Ampholines.

* The chromatograms of Servalytes (Fig. 7) show less distinct peaks than those of Ampholines. Thus, Servalytes have in general a more homogeneous molecular weight distribution. This is expected as Servalytes are prepared by introducing, in addition to carboxylic acid, also phosphonic acid and sulphonic acid groups into polyamines. Pharmalytes (Fig. 8) apparently have the most homogeneous molecular weight distribution of the three systems compared, which reflects the use of many different compounds in the synthesis.

TABLE II

DISSOCIATION CONSTANTS OF TWO PEHA ISOMERS AND ISOELECTRIC POINTS OF AMPHOLYTES DERIVED THEREFROM

PEHA isomer	*Temperature (°C)*	*Dissociation constant of amine groups*				*Isoelectric point of ampholyte with n propionic acid groups*		
		pK_1	pK_2	pK_3	pK_4	*n=1*	*n=2*	*n=3*
α	25	9.9	9.1	7.9	4.3	9.5	8.5	6.1
σ	20	10.2	9.7	9.1	8.6	10.0	9.4	8.9

Figs. 9 and 10 confirm the correctness of this hypothesis. In fact, almost all of the peaks appearing in the chromatograms of the most extreme pH ranges in these figures have elution volumes different from those of the PEHA·*x*PA system, present in the patterns of Ampholines with p*I* values between 3.5 and 10 (Figs. 3 and 6). Hence, these "new" peaks represent "new" ampholytes, not belonging to the "normal" family. Some of these "new" peaks can be identified. Thus, the prominent peak marked 7 in Fig. 10 may be assigned to methylaminopropanetricarboxylic acid (molecular weight 205). The eight steric isomers of this ampholyte, together with the seventeen isomeric amino-*n*-butanetricarboxylic acids and the eight aminoisobutanetricarboxylic acids, all obey the condition[17] that the carboxylic acid groups are close together, thereby providing the ampholytes with a low isoelectric point. The two peaks marked 8 and 9 in Fig. 9 are probably due to diaminopentanecarboxylic acid (molecular weight 146) and diaminoheptanecarboxylic acid (molecular weight 174), respectively. Many of the isomers of these ampholytes obey the condition[17] that the primary amine groups are far apart, thereby providing the ampholytes with a high isoelectric point. On the basis of these assignments, the points for peaks 7, 8 and 9 are tentatively included in Fig. 2.

CONCLUSIONS

(1) The resolution of Ampholine constituents by Bio-Gel P-4 is higher than that by the Sephadex G-15 and G-25 gels.

(2) The constituents of acidic and neutral Ampholine pH ranges associate at concentrations of about 2% (w/v). The neutral Pharmalyte pH range contains high-molecular-weight associates at even smaller concentrations.

(3) The molecular weight of carrier ampholytes generally increases with decreasing isoelectric point and, at constant isoelectric point, in the order Servalytes $<$ Ampholines $<$ Pharmalytes.

(4) The homogeneity of the molecular weight distribution increases in the order Ampholines $<$ Servalytes $<$ Pharmalytes. Presumably this is also the order of increasing number of different ampholyte species per pH unit.

(5) Normal-range Ampholines ($3.5 < pI < 10$) contain a large proportion of ampholytes derived from only a few polyamines, probably the isomers of pentaethylenehexamine.

(6) Extremely acidic and basic Ampholines ($3.5 > pI > 10$) contain large amounts of ampholytes derived from polyamines different from those of which the normal-range Ampholines are derived.

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