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SYNTHESIS OF HIGHLY DIVERSIFIED CARRIER AMPHOLYTES

EVALUATION OF THE RESOLVING POWER OF ISOELECTRIC FOCUSING IN THE Pi SYSTEM (ALPHA-1-ANTITRYPSIN GENETIC POLYMORPHISM)

R. CHARLIONET, J. P. MARTIN, R. SESBOÜÉ, P. J. MADEC and F. LEFEBVRE
INSERM (U-78) sur la Génétique des Protéines Humaines, 76230 Bois-Guillaume (France)
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SUMMARY

The use of condensing reagents such as epoxypropanol, diepoxyoctane, acrylamide and N,N'-methylenebisacrylamide in the synthesis of carrier ampholytes increased the diversity of amphoteric components. The quality of these synthetic carrier ampholytes has been tested in the separation fo variants of alpha-1-antitrypsin, a genetic polymorphism called the Pi system. A resolving power of the order of 0.005 pH unit was obtained.

INTRODUCTION

Isoelectric focusing, a separation method for amphoteric molecules, is being employed more and more by investigators in a wide variety of fields. One of the more successful uses is the demonstration of genetic polymorphism of certain proteins. At present our main concern in this aspect is the Pi system (the different allotypes of human alpha-1-antitrypsin). The resolving power of isoelectric focusing, which is of prime importance for revealing slight electric differences between similar allelic products, depends mainly on the quality of the carrier ampholytes[1,2]. Two proteins can be completely separated only in the presence of other ampholytes with isoelectric points intermediate between the proteins and with good buffering capacity. These carrier ampholytes must fulfil certain criteria. They must have (1) adequate buffering capacity in the isoelectric state to determine the pH gradient even in the presence of proteins; (2) good conductivity at their p*I*'s; (3) sufficient solubility in water.

Vesterberg[3] synthesized a number of homologues and isomers of polyamine–polycarboxylic acid; these carrier ampholytes are now available commercially under their trade name Ampholine (LKB, Bromma, Sweden). Other carrier ampholytes are now also commercially available (Servalyt, Pharmalyte, Biolyt). Synthetic procedures were described in detail by Vinogradov *et al.*[4], Righetti *et al.*[5] and Grubhofer and Borja[6]. These reports represent real progress in the development of isoelectric focusing, for

they enable the synthesis of carrier ampholytes in one's own laboratory, the selection of the pH zone required for the study, and possibly improvement in their quality.

The now classical synthetic procedure involves treating aliphatic polyamine with acrylic acid. The primary and secondary amino groups can be added to the double bonds of the unsaturated acid so that a very large number of different polyaminoacids are obtained. We have introduced an additional variation by the use of condensing reagents such as epoxypropanol, diepoxyoctane, acrylamide and N,N'-methylenebisacrylamide, in the attempt to form, after the addition on acrylic acid, a highly heterogeneous mixture of amphoteric components with a good repartition of the dissociation constants (pK) and with different isoelectric points.

Our aims were (1) to synthesize large amounts of carrier ampholytes, which would permit us to achieve better separation of the allotypes of alpha-1-antitrypsin polymorphism (Pi system) than would the commercially available ones and (2) to undertake genetic studies on a large scale. At least 24 alleles of the Pi system have been described, each of them characterized by a pattern of two major bands of focusing between pH 4.2 and 4.9. The isoelectric points of some of these alleles are very close to each other (*e.g.* PiM1, PiM3, and PiM2). Their identification is therefore very difficult if adequate carrier ampholytes are not used. Furthermore, new alleles may be disclosed if the resolving power of the method is improved.

We shall give in this report the whole synthetic procedure for the carrier ampholytes. Their quality will be described with reference to protein separation in the Pi system.

EXPERIMENTAL

Materials

The chemical reagents and their sources were: triethylenetetramine (TETA) and tetraethylenepentamine (TEPA) from Aldrich (Beerse, Belgium); a mixture of hexamines called "N6" from BASF (Levallois Perret, France); 2,3-epoxypropanol-1, 1,2-7,8-diepoxyoctane, acrylic acid, riboflavin and sucrose from Merck (Darmstadt, G.F.R.); acrylamide and N,N'-methylenebisacrylamide (Bis) from OSI (Paris, France); trichloroacetic acid from Prolabo (Paris, France); Coomassie brilliant blue from Serlabo (Paris, France). Human sera were obtained from volunteers (Blood Transfusion Centre, Bois-Guillaume, France).

The polyamines used have amino groups two methylene groups apart for TEPA and TETA, and two or three methylene groups apart for the mixture of BASF hexamines. The pentaethylenehexamine which is often described as the basic amine for the synthesis of ampholytes was not employed in this study as we were not able to purchase it.

The polyamines must be heated (70°) under vacuum (20 mmHg) for 1 h just before use, because it is crucial to operate with carbonate-free polyamines. Bisacrylamide is recrystallized from acetone, and acrylamide from chloroform, as described by Loening[7]. Acrylic acid has to be distilled just before use under nitrogen and under reduced pressure in order to remove the polymerization inhibitor.

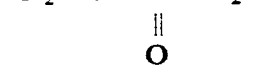
Methods

The condensing reagents are:

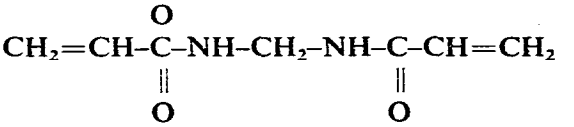
2,3-epoxypropanol-1

1,2-7,8-diepoxyoctane

acrylamide

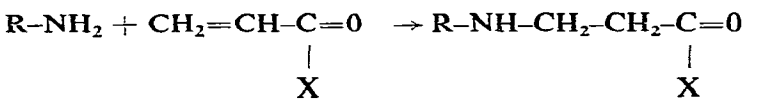


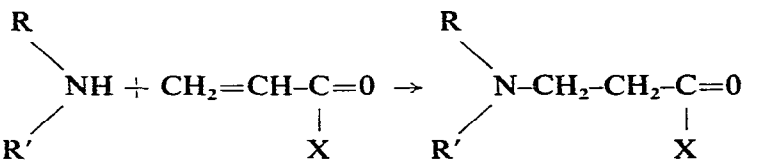
N,N'-methylenebisacrylamide



They were chosen for the facility of the reaction with amino groups and their hydrophylic properties.

Reaction of acrylamide, bisacrylamide and acrylic acid. The nucleophilic groups of the polyamines can be added at the β-carbon atom of the α,β-unsaturated acid or amide[8]:

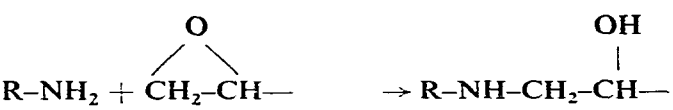




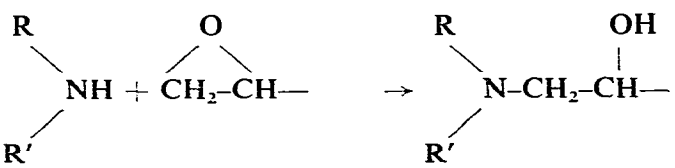
(X is OH or NHR)

We followed the reaction procedure described in detail by Righetti *et al*[15]. It is carried out in a four-necked flask equipped with a capillary for nitrogen flushing, a condenser fitted with a gas outlet and a stirrer. The polyamimes are diluted with the same volume of deionized water. Acrylic acid or a 30% acrylamide aqueous solution is added dripwise and Bis is added spoonwise. After the addition of reagents (1 h), the reaction is continued at 70° for 20 h.

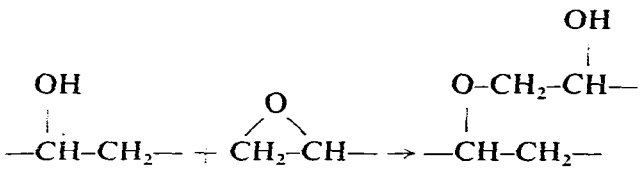
Reaction of epoxypropanol and diepoxyoctane. The reaction of an epoxide group with a polyamine is expected to follow the reaction sequence[9]:



(1)



(2)



(3)

The reaction with a primary or secondary amine leads to the formation of a secondary hydroxyl group and respectively a secondary or tertiary amine (reactions 1 and 2). Another possible reaction is between the hydroxyl and an epoxide leading to crosslinking via formation of a new secondary hydroxyl group (reaction 3). However, Shechter *et al.*[10] concluded that the alcohol–epoxide reaction does not occur to a detectable extent.

The addition of polyamines to epoxide is carried out in the same flask as described above, under nitrogen and with continuous stirring. Epoxypropanol and diepoxyoctane are added dropwise and very slowly to the aqueous solution of polyamines. The reaction is continued at 50° for 20 h.

Preparation of ampholytes. The detailed method used to prepare ampholytes from polyamines, acrylic acid and condensing reagents, can be illustrated by the following three examples. In each one, the nitrogen/carboxyl ratio is *ca.* 7:5, in order to enhance the synthesis of ampholytes at pH 5.

Ampholytes I: (TEPA, TETA, N6) + (acrylic acid). Acrylic acid (44 g, 0.61 *M*) is added dropwise for 1 h, under an inert atmosphere, to a mixture of TEPA (20 g, 0.105 *M*), TETA (7.5 g, 0.05 *M*), N6 (10 g, 0.04 *M*) and deionized water (40 ml). The reaction mixture is heated under continuous stirring at 70° for 20 h. After cooling at room temperature, enough deionized water is added to make a 40% (w/v) solution of ampholytes. They are stored in sterilized inactinic* bottles at 4°.

Ampholytes II: (TEPA, TETA, N6) + (acrylamide) + (Bis) + (acrylic acid). A 30% solution in deionized water of acrylamide (50 ml, 0.21 *M*) is added dropwise for 1 h and under inert atmosphere to a mixture of TEPA (20 g, 0.105 *M*), TETA (7.5 g, 0.05 *M*), N6 (10 g, 0.04 *M*). The reaction continues under stirring at 70° for 4 h. The mixture is then cooled and Bis (6.5 g, 0.042 *M*) is added spoonwise; the reaction mixture is heated to 70° for 16 h. Acrylic acid is then added and the ampholytes stored under the same conditions as above.

Ampholytes III: (TEPA, TETA, N6) + (epoxypropanol) + (diepoxyoctane) + (acrylic acid). Epoxypropanol (15 g, 0.21 *M*) is added dropwise, very slowly, under an inert atmosphere, to a mixture of TEPA (20 g, 0.105 *M*), TETA (7.5 g, 0.05 *M*), N6 (10 g, 0.04 *M*) and deionized water (40 ml). The reaction continues under stirring at 50° for 4 h. The mixture is then cooled and diepoxyoctane (6 g, 0.04 *M*) added dropwise. The reaction mixture is heated to 50° for 16 h. Acrylic acid is then added and ampholytes stored under the same conditions as above.

Fractionation of carrier ampholytes. Only ampholytes focusing between pH 4.2 and 5.4 are useful for the study of the Pi system. They are isolated by a preparative isoelectric focusing technique in open horizontal layers of granulated gel, as described by Radola[11].

The granulated gel used as the stabilizing medium is Sephadex G-15 (Pharmacia, Uppsala, Sweden). This is preferred to Sephadex G-75 (or G-100) superfine

* inactinic = not permitting the chemical action of radiant energy.

used in preparative protein because of its remarkable mechanical properties. We used the electrofocusing kit for granulated gel, the Multiphor and the model 2103 power supply from LKB.

The fractionation procedure is as follows. A slurry of Sephadex G-15 (100 ml) is filtered on a glass filter funnel and washed with deionized water. 25 ml of a 40% solution of synthetic ampholytes is mixed with the filtered gel in a beaker. The homogenized suspension is then poured on to a horizontal tray with three layers of wet strips at each end. The suspension spreads evenly. The excess of water is evaporated with a light stream of air. At the end of this process (which is easy to determine with Sephadex G-15), the cathode and anode strips, respectively soaked with sodium hydroxide (1 *M*) and phosphoric acid (1 *M*), are positioned and the tray is put on the Multiphor unit. The limiting electrical conditions are 11 W, 20 mA, 1500 V. A run of 48 h is needed for a good separation, after which a grid allows the focused ampholytes to be collected. Each separated zone is put in a vial and resuspended with deionized water. The pH is then measured with a combined glass microelectrode fitted on a digital pH meter (Metrohm Herisau). The ampholytes can be eluted very easily by filtration. They are concentrated on a rotative evaporator and kept as 40% solution (w/v) in sterilized inactinic bottles at 4°.

In this study, we used carrier ampholytes encompassing three pH ranges: 4.2–5.3, 4.3–4.9 and 4.4–4.7. The larger ranges were separated directly from the solution of synthetic ampholytes. The narrower one required further fractionation from ampholytes of pH range 4.2–5.3. The pH range 4.3–4.9 was also obtained by fractionating commercial ampholytes (Ampholine LKB, 3.5–5.0).

Analytical isoelectric focusing on polyacrylamide gel plate. This is performed on a LKB Multiphor apparatus. The gel is made to a final concentration of acrylamide 5%, carrier ampholytes 1% (w/v) and sucrose 12% (w/v). Polymerisation is accomplished with riboflavin 0.04% (v/v) and UV light for 1 h. A prerun is carried out for 1 h and, after application of the samples, isoelectric focusing is performed for 2 h with the following maximal electrical conditions: 10 W, 10 mA and 1600 V. The pH gradients in the gel slabs are measured with a contact electrode (Ingold, Sofranie, France) and read on a digital pH meter. Fixation with trichloroacetic acid, staining with Coomassie blue and destaining are carried out as described by Vesterberg *et al.*[12].

Conductivity measurements. These were carried out, as described by Righetti *et al.*[13], to compare the conductivity as a function on the pH gradients between synthetic and commercial carrier ampholytes. After focusing, the gels were cut carefully into $6 \times 0.5 \times 0.1$ cm slices, and each fraction was eluted for 4 h in 4 ml of distilled water. Conductivity measurements were made with a Metrohm Herisau conductimeter E382 (Sofranie, France). The pH measurements of each fraction were made with a combination electrode (Ingold).

RESULTS

Fig. 1 shows the pattern of several alpha-1-antitrypsin phenotypes (BM3, IM2, M1S, M1Z, Z, S, M1M3, M1M2, M2M3) obtained with ampholytes I (Fig. 1a) and ampholytes III (Fig. 1b). Both ampholytes have been fractionated to give pH gradients in the range 4.2–5.3.

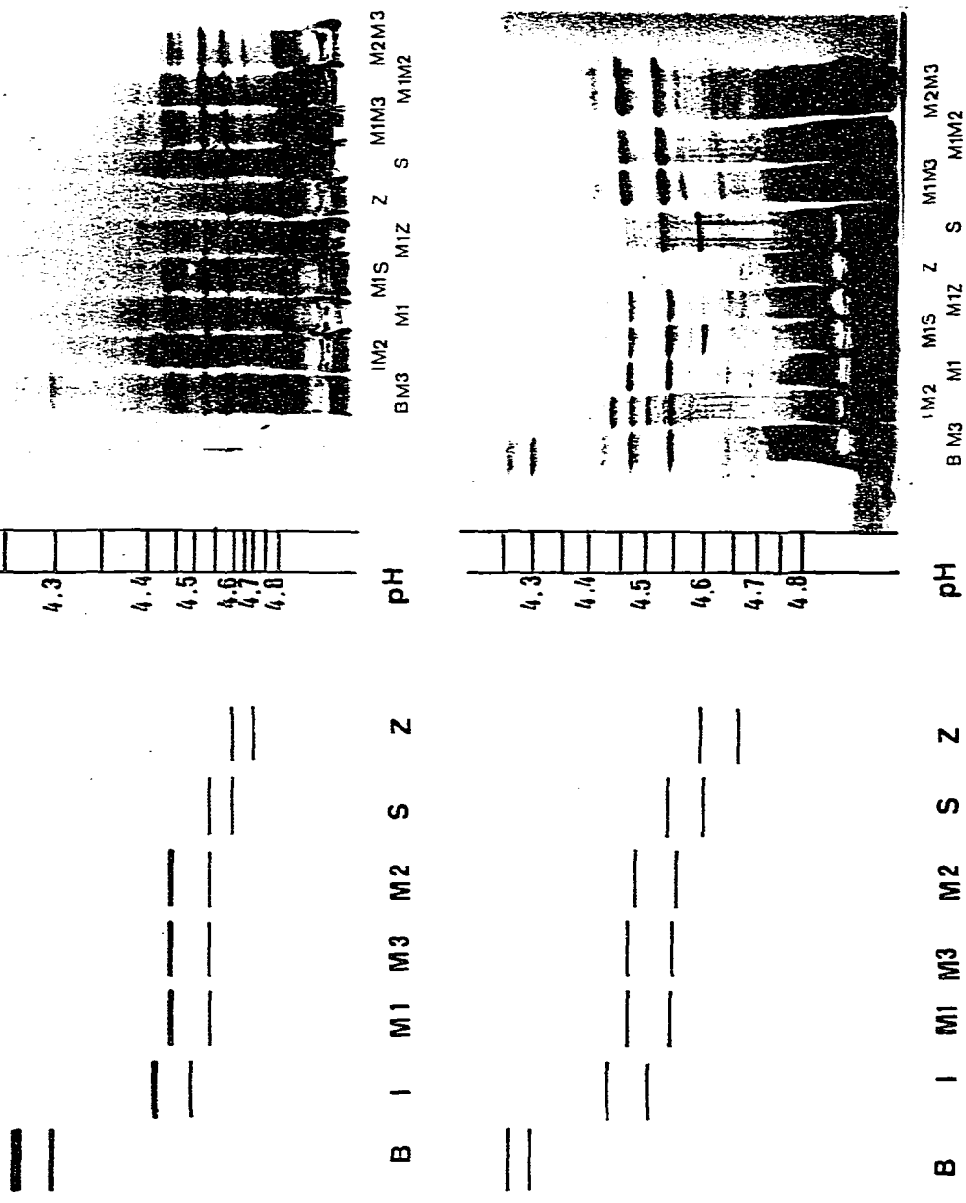


Fig. 1. Results obtained with (a) ampholytes I and (b) ampholytes III, following fractionation to give pH gradients in the range 4.2–5.3. On the right are the isoelectrofocusing patterns of several alpha-1-antitrypsin phenotypes. In the middle is the pH scale as measured by a contact electrode on the polyacrylamide gel plate after focusing. On the left are the graphs of the two major bands (namely the 4 and 6 bands) of the alpha-1-antitrypsin patterns for several alleles.

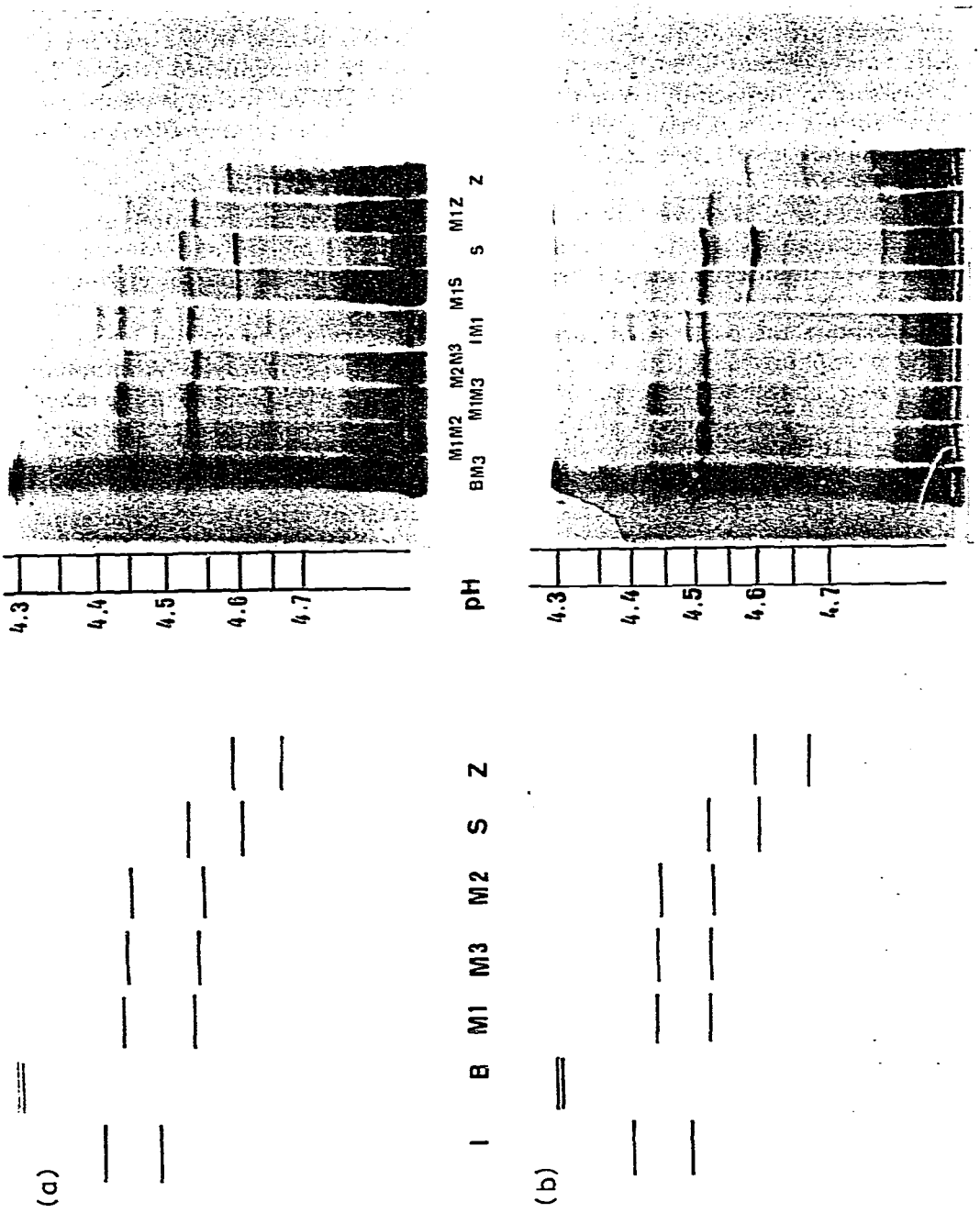


Fig. 2. Results obtained with (a) ampholytes III and (b) commercial ampholyte (Ampholine LKB), following fractionation to give pH gradients in the range 4.3–4.9. The details are the same as for Fig. 1.

It is evident that ampholytes I are quite inadequate for determining alpha-1-antitrypsin phenotypes. There are gaps in distribution within the pH range 4.2–5.3, the pH gradient is not linear, and the quality seems to be very poor. It can be seen that some bands are not at all well focused, *e.g.* the M_4 bands. In the pattern of each allele, bands '4' and '6' are separated but cannot be distinguished from other proteins focusing in the vicinity. When ampholytes III are used, the gaps disappear, the pH gradient becomes nearly linear, and the quality of carrier ampholytes seems improved. (Ampholytes III give the same results as ampholytes II.)

Synthetic ampholytes III can be used for the determination of the alpha-1-antitrypsin phenotypes. However, from this zone of ampholyte fractionation, a clear-cut distinction cannot be made between the M1 and M3 or M3 and M2 alleles. Only M1 and M2 are distinguished. This explains the necessity of a narrower zone of fractionation of the ampholytes.

Fig. 2 shows the patterns of several alpha-1-antitrypsin phenotyes obtained from synthetic ampholytes III (Fig. 2a) and from commercial ampholytes (Ampholine LKB) (Fig. 2b), each following further fractionation to give pH gradients in the range 4.3–4.9. The separations of the 4 and 6 bands of M1, M3, and M2 are excellent for ampholytes III. In the same manner, S_4 and M_6 bands, Z_4 and S_6 bands are very clearly distinguished. The resolving power reached by ampholytes III in this case can be estimated at 0.005 pH units or less. The commercial product used in the same pH range does not give as good separtion as ampholytes III (Fig. 2b).

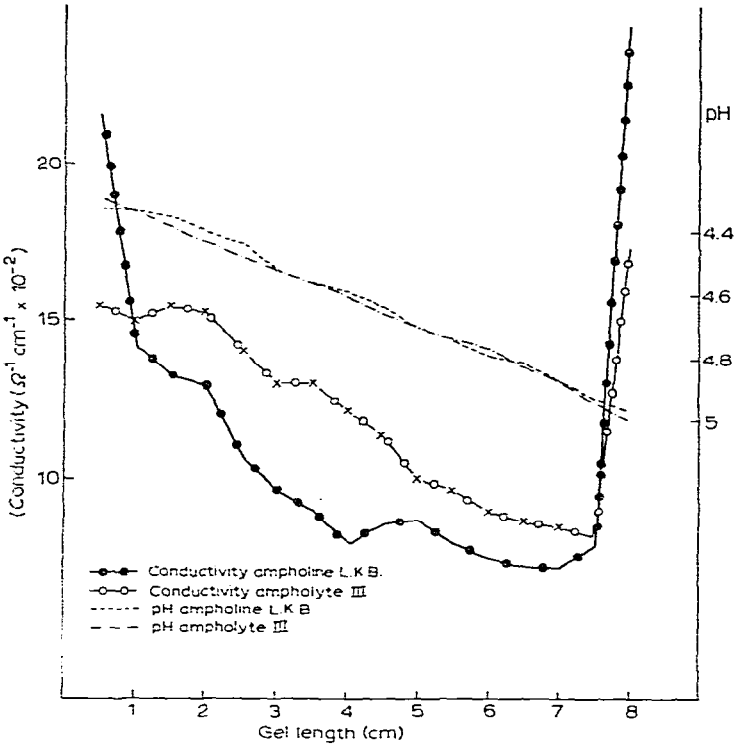


Fig. 3. Comparison of the conductivity and the pH gradient profile for ampholytes III and Ampholine LKB, fractionated in the pH range 4.3–4.9.

Some physicochemical data relating to ampholytes III and Ampholine (LKB), both of them fractionated in the pH range 4.3–4.9, are compared in Fig. 3 (conductivity and pH gradient profiles). Ampholytes III have a slightly better conductivity throughout the pH gradient considered than Ampholine LKB. When ampholytes III are subjected to a second step of purification to obtain the very narrow pH range 4.4–4.7, the separations of M1, M2 and M3 phenotypes are impressive (Fig. 4). Furthermore, some new alleles are revealed. Although the identification of certain of these PiM heterozygotes is unreliable, the corresponding new alleles are distinguishable when focused side by side. The differences between their p*I*'s can be estimated to be less than 0.001 pH unit. The family studies of these new variants will be published elsewhere.

DISCUSSION

The resolving power

Synthetic ampholytes III used in optimum conditions can separate proteins whose p*I*'s are less than 0.005 pH unit apart (*e.g.*, $M1_6$ and $M3_6$ or $M2_6$ and $M3_6$) and allows us to distinguish proteins differing only by 0.001 pH unit. Such a resolving power has not been realized previously for the Pi system.

Allen *et al.*[14] determined alpha-1-antitrypsin phenotypes with isoelectric focusing on polyacrylamide gel slabs containing commercial carrier ampholyte (Ampholine LKB 3.5–5.0). They were able to separate the basic variants and to measure their isoelectric points. Since then, this method has been widely used. Nevertheless, the resolving power of this technique was without doubt overstated when Allen claimed that proteins with p*I*'s differing by 0.0025 pH unit could be resolved. In fact, M1 and M2, which differ by only 0.01 pH unit, were not distinguished.

The separation of M1 and M2 was initially reported by Frants and Eriksson[15], and M3 was revealed a few months later by one of us[16] and confirmed recently by Frants *et al.*[17]. These improvements in resolving power were realized with Ampholine LKB by using several methods of decreasing the slope of the pH gradients. Two of them have been used in our laboratory: the mixture of Ampholine LKB 4–6 and 3.5–5.0, and the fractionation of Ampholine LKB 3.5–5.0. This study shows that our synthetic carrier ampholytes III compare favorably with Ampholine LKB in physicochemical properties and in the resolving power.

Frants *et al.*[17] have chosen another interesting way to influence the slope of the pH gradient: the addition of amphoteric substances called 'separators' by Caspers and co-workers[18] to separate M1, M2 and M3. These proteins have also been recently claimed by Kueppers and Christopherson[19] to be separated with Ampholine LKB 3.5–5.0. However, it seems to us that they misinterpreted the phenotypes of the serum used. On the one hand, the distance between the so-called $M2_6$ and $M1_6$ bands is far too big in comparison with the distance between $M1_6$ and $M1_4$ bands. On the other hand, the excess of carrier ampholyte they used would broaden the focused bands rather than improve the resolving power.

Increasing the diversity of carrier ampholyte species

The gaps in the distribution of ampholytes I, which are seen in the pH range 4.2–5.3, are filled when ampholytes II and III are used. The introduction of epoxy-

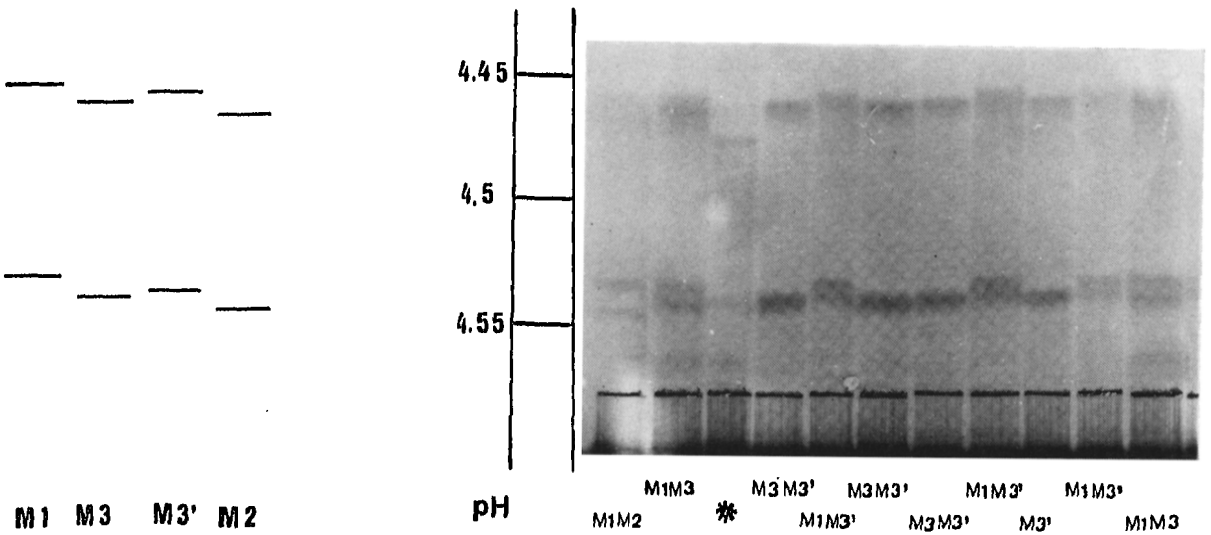
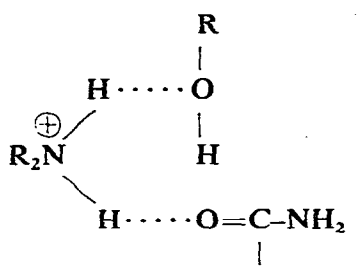


Fig. 4. Results obtained with ampholyte III subjected to a second step of purification to obtain the pH range 4.4–4.7. The details are the same as for Fig. 1. One of the new variants discovered can be seen; we have provisionally called it M3′. Although the identification of the Pi M3M3′ heterozygotes is unreliable, the corresponding alleles are distinguishable when focused side by side. The asterisk refers to an old serum previously phenotyped Pi M3′.

propanol (or acrylamide) and diepoxyoctane (or bisacrylamide) is sufficient for the synthesis of new species of ampholytes with evenly distributed isoelectric points and with a good repartition of the dissociation constants. Two explanations may be put forward to justify this increase of diversity of ampholyte species.

The main polyamine used in this study is TEPA, which is a pentamine with seven nucleophiles (>N-H). The number of acrylic acid molecules to be coupled to one molecule of TEPA for the synthesis of ampholytes having isoelectric points in the vicinity of pH 5 might be estimated at 4. From a strictly formal point of view, there are theoretically ten different ampholyte species and 24 different substrates for further addition reactions, which can be synthesized using these conditions. Neutral reagents such as epoxide or acrylamide are added. They will not notably modify the isoelectric point of the ampholytes, so that we can continue to consider the ratio carboxylic/TEPA to be 4. With the quantity of epoxypropanol (or acrylamide) added (epoxide/TEPA=1), it might be expected that mainly three ampholyte populations would be synthesized: ampholytes having reacted with zero, one or two molecules of epoxypropanol. Again from a strictly formal point of view, we could theoretically get a total of 58 different ampholyte species, and 89 different substrates for further addition reations. If we now consider the addition of a coupling reagent such as diepoxyoctane (or bisacrylamide), in the ratio diepoxyoctane/TEPA =1/5, the most probable configuration we can obtain for the molecules that have reacted is the crosslinking of two TEPA by one dialcohol–octane bridge. This would lead to 3916 different ampholyte species. This number has been calculated from C_2^{89}, which represents the possible set of two things selected from 89, irrespective of arrangement within the set. In fact, we use a mixture of several polyamines in the synthesis of ampholytes; thus other combinations could be envisaged, so that the number of different ampholyte species with isoelectric point near pH 5 should be much greater than 3916. However, this number should be considerably reduced by the fact that, in TEPA for instance, the seven nucleophiles do not possess the same reactivity. The probability that some of them may react will be very low, so that the corresponding ampholyte species should be practically non-existent. It should also be kept in mind that most of the different synthesized ampholyte species, although not quite identical in configuration, should have nearly indistinct isoelectric points.

The increase of diversity of ampholyte species might also be explained by the possibility that the introduction of the alcohol or amide groups changes the strength of the nitrogenous bases. This could be due to the fat that the basic strength of an amine is determined, among other factors, by the extent to which the cation, formed by uptake of a proton, can become stabilised. In a molecule in which hydroxyl (or amide) groups are present together with amino groups, such a stabilisation is possible possible via hydrogen bonding in the following way[20]:



The effect of this intramolecular hydrogen bonding on the constants of dissociation of the basic groups and on the isoelectric points of the final carrier ampholytes is difficult to quantify. Furthermore, hydroxyl (or amide) should compete with carboxylic groups in the formation of hydrogen bonds. Nevertheless, the fact that both kinds of reagents, epoxypropanol and diepoxyoctane (or acrylamide and bisacrylamide), are necessary to fill the gaps indicates that the two proposed explanations are complementary.

It should also be noted that the addition of epoxypropanol and diepoxyoctane has to be carried out before the addition of acrylic acid, otherwise the quality of ampholytes is not improved enough. This is somewhat surprising since we could have expected another type of reaction between the epoxide and the carboxylic groups, leading with tertiary amine as catalyst to an hydroxyl ester.

CONCLUSION

We have tried to improve the quality of carrier ampholytes. The introduction of coupling reagents such as epoxypropanol, diepoxyoctane, acrylamide and bisacrylamide during their synthesis has proved to be useful. However, the polyamines employed are not the best for synthesizing ampholytes. It has been shown that TEPA and TETA on their own give very poor mixtures of ampholytes[3,5]. The ampholytes obtained with the mixture of hexamines 'N6' provided by BASF do not appear to be better than TEPA ampholytes, and we have confirmed that our ampholytes I are not suitable for the determination of the Pi system by isoelectric focusing. Curing of pentaethylenehexamine (which has proved to be very good for ampholyte synthesis) with the coupling reagents used in this study should give excellent results. Unfortunately, it is now practically unavailable.

Other amines could be tried for this purpose. Condensation of ethyleneimine with propylenediamine[6] could give a very good starting material. Other approaches to the improvement of the properties of carrier ampholytes could be achieved by looking for different acids (itaconic acid[13], propanesulphone and chloromethylphosphoric acid[6]) or other coupling reagents.

It is worth noting that in this study the synthesis of ampholytes has been conducted in such a way as to favour ampholytes with isoelectric points in the vicinity of pH 5. The synthesis of carrier ampholytes of different isoelectric points should be carried out in different conditions: the nature and the proportion of the reagents must be adapted empirically. We wish to add that this necessary adaptation can easily be made in a standard biochemical laboratory.

We succeeded in synthesizing large amounts of carrier ampholytes, which allowed us to study the Pi system more thoroughly than did the commercially available ones. The improvement of resolving power is of prime importance to reveal new alleles. We are convinced that the limit of isoelectric focusing as the method for protein separation has not yet been reached. Further research on the chemical nature of ampholytes must be undertaken. Synthetic carrier ampholytes fractionated in narrow pH range by means of preparative electrofocusing might be considered as quite versatile 'separators'.

ACKNOWLEDGEMENTS

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