ACTA CHEMICA SCANDINAVICA 23 (1969) 2653–2666

Synthesis and Isoelectric Fractionation of Carrier Ampholytes

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The method for isoelectric focusing and separation of ampholytes such as proteins, in stable pH gradients, requires special low molecular ampholytes with a good buffering capacity. Such ampholytes, called carrier ampholytes, are characterized by closely spaced pK values. Lack of suitable ampholytes has for several years prevented widespread use of this method.

Coupling of carboxylic acid residues to polyethylene polyamines gives series of homologues and isomers of polyamino polycarboxylic acids with different isoelectric points, pI. Acrylic acid is brought to react with different polyethylene polyamines in a water solution. The yield of ampholytes is then nearly 100 %. The ampholytes are focused and fractionated in a multicompartment electrolyzer, into groups with closely spaced pI values.

The yields, the buffering capacities, and the conductivities of the fractions are determined. The pK values of the ampholytes obtained with ethylene diamine are estimated from the titration curves. The pK-values of the amines seem to shift only slightly upon substitution with a carboxylic acid residue. The pK values and thus also the pI values of the ampholytes are therefore predictable to a high degree. The pI's of the ampholytes will thus be in the pH range between the pK of the most acidic carboxyl group with pK at about 3, and the pK of the most alkaline amino group, with pK at about 10. With amines having pK values closer than 3 pH units, ampholytes with a good buffering capacity are obtained. Among the amines examined, pentaethylene hexamine is most suitable.

These carrier ampholytes have many valuable properties:
1) they are a mixture of many isomers and homologues with different, closely spaced pK and pI values, covering the pH range from 3 to 10;
2) their buffering capacity is as good as or better than that of histidine, which is considered a good carrier ampholyte. This property and the distribution of their pI, means that they can dictate pH courses in the pI range of most proteins;
3) the conductivity course as caused by them, and thus also the field strength distribution between the electrodes during isoelectric focusing, is satisfactory;
4) their solubility in water is very good;

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5) in a 1 % solution their light absorption above 260 nm is low. This is important when light absorption measurement is to be used for the detection of proteins focused in a pH gradient.

Numerous applications in focusing and separation of proteins, made possible with these polyamino polypropionic acid mixtures, have already demonstrated the value of these carrier ampholytes. A patent on the synthesis and the fractionation of carrier ampholytes is pending.

The theory for isoelectric focusing and separation of proteins with the aid of carrier ampholytes has been presented by Svensson.[1,2] A brief summary will be given here.

Ampholytes are characterized by the number of protolytic groups and their corresponding pK-values. For low-molecular ampholytes, the number of protolytic groups and their respective pK values can be determined by acidimetric titration. The resulting titration curve, giving the amount of protons bound to or released from the ampholyte as a function of pH, also represents the variation of the electric charge with pH provided that there is no salt interaction. As the electric mobility is roughly proportional to the charge of a molecule, the mobility-pH curve also becomes correlated to the titration curve.

The pH where the electric charge of an ampholyte is zero is called its isoionic point. For a biprotic ampholyte, it is given by the formula:

$$\tfrac{1}{2}(\mathrm{p}K_1+\mathrm{p}K_2) \qquad (1)$$

The same formula can be applied for oligoprotic ampholytes using the two pK's closest to the isoionic point, provided that other pK's are not too close to it.

The pH where the electric mobility is zero is called the isoelectric point (pI). Under the conditions given above, it is identical with the isoionic point, and in practice it is generally not very far from it.

The contribution to conductance given by an ampholyte in solution is proportional to the concentration of ions except zwitterions. At the pI, there are always large amounts of zwitterions, but there are also appreciable quantities of cations and anions if pK_1 and pK_2 are close together. Ampholytes being isoelectric between two closely spaced pK values have consequently a good conductance in the isoelectric state.

The buffering capacity is proportional to the slope of the titration curve. At the isoelectric point, therefore, there can be a good buffering capacity only in the case of two overlapping dissociation steps, that is, if the two pK's on either side of the pI are not very far apart.

To sum up, ampholytes isoelectric between two closely spaced pK values possess a good conductance as well as a good buffering capacity in the isoelectric state. If more than two pK values are closely spaced around the pI, these two properties of the isoelectric ampholyte are still further improved. Low-molecular ampholytes of this kind are called carrier ampholytes because of their usefulness in isoelectric focusing of proteins.[2] For carrier ampholytes the numerical value of pK_2−pK_1 should not be greater than 3 pH units,

a condition that excludes practically all biprotic ampholytes, *e.g.* the neutral amino acids.

On convection-free electrolysis of a water solution of carrier ampholytes with different pI's, each ampholyte will be focused at its own pI. The more acid ampholytes, with lower pI's, will be focused near the anode and the more alkaline ones, with higher pI's, near the cathode. The carrier ampholytes dictate the pH at the point of focusing due to their buffering capacity. In the electrolysis cell, the pH will thus show increasing values from anode to cathode, that is, a pH gradient in the direction of the current will be established. The extension of the pH range will be determined by the most and least acidic ampholytes including water, which is, of course, an ampholyte although no carrier ampholyte. If salts are also present, the corresponding acids and bases will collect at the electrodes, giving rise to an extended pH range. The shape of the pH course at a steady state depends on the pI's, the concentrations, and the electrochemical properties of the carrier ampholytes.

The conductance course is as essential as the pH course since it determines the field strength distribution in the electrolysis cell through the equation:

$$E = i/\varkappa \tag{2}$$

where i is the current density, and $\varkappa$ the conductance. Thus, in case of a greatly variable conductance, the field strength becomes uneven. Below pH 4 and above pH 10 it is no problem to obtain a good conductance already because of the abundance and high mobilities of the water ions. In the region between pH 4 and 10, and especially at the neutral point, the conductance contribution of the solvent is very slight, and consequently well-conducting carrier ampholytes are necessary in order to realize a satisfactory field strength distribution. If such ampholytes are not available, an extremely deep conductance minimum, and a correspondingly high field strength maximum, develops in the neutral range. In order to avoid excessive heating at this point, extremely low currents then have to be applied, implying very low field strengths in other parts of the pH gradient.

The degree of focusing of an ampholyte is given by the equation:[1]

$$2\ x_i = 2\sqrt{\frac{-D}{E(\mathrm{d}u/\mathrm{dpH})(\mathrm{dpH}/\mathrm{d}x)}} \tag{3}$$

where $2x_i$ is the breadth of the Gaussian distribution curve taken as the distance between the inflexion points, D the diffusion coefficient, E the field strength, $\mathrm{d}u/\mathrm{dpH}$ the (negative) slope of the pH-mobility curve at the isoelectric point, and $\mathrm{dpH}/\mathrm{d}x$ the value of the pH gradient in the direction of the current. Since proteins have low values of D and high values of $\mathrm{d}u/\mathrm{dpH}$, it is evidently possible to get a sharp focusing of proteins at their respective isoelectric points. This permits an exact determination of their pI values simply by measuring pH at their respective maximum concentrations. Vesterberg and Svensson[3] have shown that it is possible to separate even very similar proteins differing in pI by only a few hundredths of a pH unit. As the carrier ampholytes should be small molecules as compared to proteins, they may subsequently be removed from them by molecular sieving processes, *e.g.* dialysis or chromatography on Sephadex.

For permitting a general use of this method, several carrier ampholytes with pI's distributed over the pH interval between 3 and 10 must be available. Outside this interval, the pH gradient can be built up by using weak acids and bases, respectively. Extensive searching during several years for commercially available ampholytes which might be useful as carrier ampholytes has not been very successful.[2] No good ampholytes were found with pI values between 3.9 and 7.3. Unfortunately, this is just the pH range of interest for a great many proteins. By partial hydrolysis of proteins, oligopeptides with various pI values and rather good electrochemical properties could be obtained, but those isoelectric between pH 5 and 7 were still too bad.[3]

All the mentioned types of ampholytes represent a large group of most different chemicals. It would be very difficult to predict or examine their possible interactions with proteins. In order to facilitate the detection of proteins after focusing, the ampholytes should not contain peptide bonds or have light absorption above 260 nm. Furthermore, the ampholytes must be easily soluble in water. These criteria excluded many ampholytes, *e.g.* those having an aromatic structure. To sum up, most of the earlier known ampholytes are for one reason or the other not very suited as carrier ampholytes.

A THEORETICAL APPROACH TO THE SYNTHESIS OF A NEW SYSTEM OF CARRIER AMPHOLYTES

Knowing the criteria for good carrier ampholytes, one could consider to synthetise "tailor-made" ampholytes by incorporation into one molecule of an acidic and a basic group having closely spaced pK values. This way of approach is, however, connected with two serious difficulties. Firstly, there are very few monovalent protolytes dissociating in the region between pH 5 and 9. Monoprotic carboxylic acids dissociate below pH 5, and most aliphatic and alicyclic amines dissociate above pH 9. Between pH 5 and 9, there are only few substances, *e.g.* hydroxy-substituted aliphatic saturated amines and some aromatic bases. The latter are not wanted because of their high light-absorption. Secondly, on coupling together a monoprotic acid with a monoprotic base, the pK's of the resulting ampholyte in general turn out to be far from the pK's of the original molecules. This depends upon interaction between functional groups if these are present in the same molecule.[4] Such interaction will be discussed in some detail below.

Table 1. Dissociation constants at 25°C.

	pK_1	pK_2
Propionic acid [4]	4.9	
Amino ethane [7]	—	10.7
α-Alanine [7]	2.3	9.9
β-Alanine [7]	3.6	10.2

The interaction between carboxyl and amino groups in the same molecule can be illustrated by comparing the pK values of α- and β-alanine with those of the corresponding monoprotic compounds, *cf.* Table 1. As can be seen there, an amino group substituted into propionic acid lowers the carboxyl pK considerably, especially if the amino group is in the α position, whereas the influence of the carboxyl group on the pK of the amino group is much less pronounced, especially in β position.

Table 2. Dissociation constants for ethylene amines. The values are taken from Ref. 7.

Amine	pK_1	pK_2	pK_3	pK_4	pK_5	Temperature °C
Ethylene diamine	10.1	7.0				20
Diethylene triamine	9.9	9.1	4.3			20
Triethylene tetramine	9.9	9.2	6.7	3.3		20
Tetraethylene pentamine	9.9	9.1	7.9	4.3	2.7	25

The interaction between similar groups within one and the same molecule is very interesting, and can be studied on a series of polyamines, the pK's of which are given in Table 2. This table shows that the higher pK values are rather independent of the number of amino groups, whereas the pK range is extended to lower pH's for every additional amino group. Consequently, by considering some polyvalent amines, there is not difficult to find pK values well distributed in the pH range 3 to 10. By coupling to these amines one or more residues containing a carboxylic group, many ampholytes with different pI values can be obtained. The author has used an addition reaction, by which propionic acid residues are coupled in β position to amino groups. The added groups will, of course, to some extent influence the pK values of the amino groups. Almost no pK values for ampholytes of this type are available in the literature. However, one example may illustrate the resulting pK values to be expected, *cf.* Table 3. It can be seen that the pK's of the amino groups shift

Table 3. Dissociation constants at 30°C.

	pK_1	pK_2	pK_3	pK_4
Ethylene diamine [7]	9.8	6.7		
Ethylene-N,N'-dipropionic acid [8]	9.6	6.9	3.8	3.0

very little only. On the other hand, when compared with propionic acid with pK 4.9, the pK's of the carboxylic groups are lowered markedly. It is well known that mutual interaction between two carboxylic groups decreases with increasing number of atoms separating the groups.[4] Interaction between

carboxylic groups in the ampholytes will accordingly be rather weak, because they cannot come closer than one N and four C atoms. Probably most carboxyl pK values in the ampholytes will, due to interaction with the amino groups, be below pH 4. The most acidic ampholytes only will have pI values influenced by the absolute values of the pK's of the carboxylic groups. For most ampholytes the pK values of the amines are therefore more interesting. It follows from the above that the resulting pK's, and thus also the pI's of the ampholytes, are predictable to a high degree.

Supposing that we have triethylene tetramine (for pK's see Table 2), and that we add one propionic acid residue, the ampholyte thus obtained will be isoelectric at a pH between the two highest pK values of the amine, *i.e.* pK_1 and pK_2. Consequently, the pI will be about 9.6. If two propionic acid residues are added, the ampholyte will be isoelectric between pK_2 and pK_3. From these examples we see that with a given polyamine the number of introduced negatively charged groups determines the interval in which the pI has to be. The exact value is directed by the pK values in the ampholyte which are closest to the pI. In order to get many carrier ampholytes with pI's between 3 and 10 it is accordingly important to start from a collection of amines with rather closely spaced pK values distributed over the corresponding pH range. A closer inspection of Table 2 reveals that polyethylene amines containing four or more nitrogen atoms are valuable for the synthesis of carrier ampholytes. Some of the amines listed in Table 2 are, however, not quite ideal, because the distance between some of the pK values is too big, as for instance in tetraethylene pentamine between pK_3 and pK_4. But it can be predicted that higher homologues of this series of amines should have more closely spaced pK values. Unfortunately no pK values for pentaethylene hexamine could be found in the literature. The titration curve of this amine, see Fig. 1, indicates that the pK values of this amine are more closely spaced

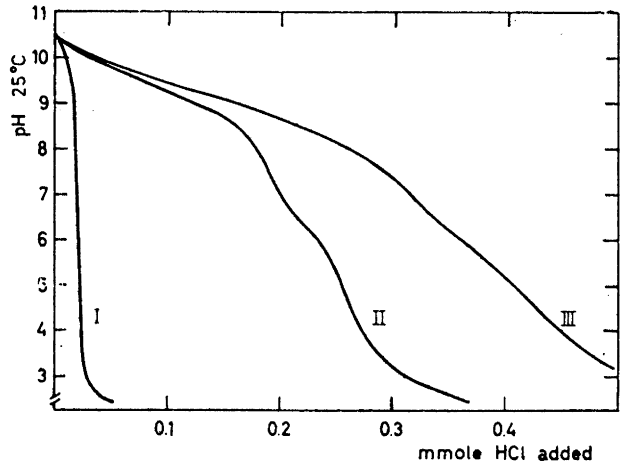

Fig. 1. Titration curves of I, water, II, 0.09 mmole of triethylene tetramine, and III, 0.1 mmole of pentaethylene hexamine respectively, at 25°C.

than in homologues containing fewer amino groups. Furthermore pentaethylene hexamine can exist in three isomers, which are most probably also present in commercial products, and these should have slightly different pK values, due to different structure, and accordingly somewhat different mutual interactions.[4] Isomers are valuable because they increase the number of different ampholytes obtainable.

The conclusion from the above is that excellent theoretical conditions are at hand for the production of a system of carrier ampholytes with a great number of members with different pI values. The pI of these will, accordingly, be in the pH range between the pK of the most acidic carboxylic group and the pK for the most alkaline amino group.

MATERIALS AND METHODS

Chemicals. Acrylic acid 99 % (AB Reagens, Gothenburg, Sweden) was distilled at 10 mm Hg immediately before use to get rid of the polymerisation inhibitor present in the commercial product. The aliphatic amines, ethylene diamine, triethylene tetramine tetraethylene pentamine, pentaethylene hexamine (Fluka, Basle, Switzerland) were distilled in high vacuum before use.

Titrator. An automatic recording Radiometer Titrigraph model TTT 1, equipped with a thermostated titration vessel kept at 25°C, was used.

Spectrophotometry. Light absorption measurements were performed in a Beckman recording spectrophotometer, model DK 2.

Synthesis of the carrier ampholytes. An addition reaction which depends on the reactivity of primary as well as secondary amino groups to $\alpha-\beta$ unsaturated carboxylic acids was used.[5] The coupling takes place preferentially on the β-carbon atom in the acid. Depending on the proportions of acid to amine, one or more carboxylic groups will be introduced into the ampholyte. The reason for the choice of this synthesis method will be dealt with more extensively in the discussion. The reaction can be illustrated by:

$$R_1-\overset{+}{N}H_2-(CH_2)_2-\overset{+}{N}H_2-R_2+CH_2=CH-COO^- \rightleftharpoons R_1-\overset{+}{N}H_2-(CH_2)_2-\overset{+}{N}H(-CH_2-CH_2-COO^-)-R_2$$

where R_1 and R_2 can be hydrogen or an aliphatic radical with additional amino groups.

The reaction was carried out in water solution at 70°C for 5 h in a three-necked flask equipped with reflux conderser, stirrer, and thermometer. The completeness of the reaction, *i.e.* disappearance of the acrylic acid, was checked on samples taken from the reaction mixture. The samples were acidified to pH 2.5 with 1 M H_2SO_4, and the acrylic acid was extracted with ether in a separatory funnel. The ether was then removed by evaporation, and the residue was dissolved in 2 ml of distilled water. This solution was titrated with 0.01 M $KMnO_4$. This test revealed that all acrylic acid was consumed after 5 h. After the reaction was completed, water was added to give 500 ml solution, which was electrofocused in a multimembrane electrolyzer.

Construction of the electrolyzer and operation thereof. An electrolysis apparatus of 550 ml capacity was used, with electrodes of platinum wire-netting at both ends, and the space between was divided by membranes of polyvinylchloride paper into 20 compartments.[6] (Figs. 2a and 2b). Into each cell narrow glass tubes were inserted, through which cooling water was circulated. The cells were also equipped with a stirring device to increase the efficiency of the cooling and to conteract temperature and concentration gradients within the cells. There was little risk for transport of liquid from one cell to another because the movements in all the cells were equal and well synchronized. The apparatus will be described in detail in a forthcoming paper.

Ordinarily a 4 to 8 % solution of ampholytes was electrolyzed. The carrier ampholyte solution was filled into the cells 3 to 19. In order to prevent the ampholytes from contact

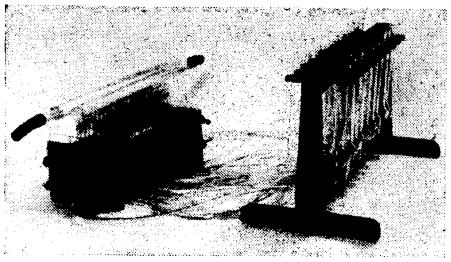

Fig. 2a. Photograph of a multicompartment electrolyzer for isoelectric focusing and separation of ampholytes. To the left: electrolyzer. To the right: a fraction collecting system.

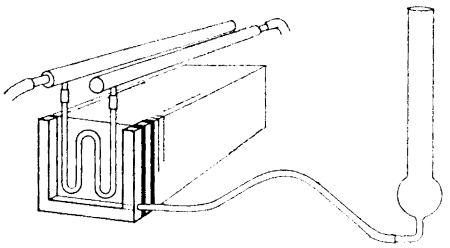

Fig. 2b. Schematic drawing of the apparatus shown in Fig. 2a, but with the first cell removed.

with the anode, cells 1 and 2 were filled with 50 ml of 0.1 M H_2SO_4. If this precaution was not taken, the ampholyte solution got a yellow colour, spreading from the anode.

In order to prevent the ampholytes from contact with the cathode, cell number 20 was filled with 25 ml of 0.1 M NaOH. However, no chemical changes of the ampholytes have been observed at the cathode.

The electrolysis was carried out for 24 h with a maximum load of 500 W. The potential was increased to 800 V at the end. The current was then switched off and the cells were emptied simultaneously through a tube in the bottom of each cell into fraction collection tubes, by raising the electrolyzer above the level of the tubes. The tubes were then placed in a water bath with a temperature of 20°C before pH was measured. The yields were determined by dry weight analysis.

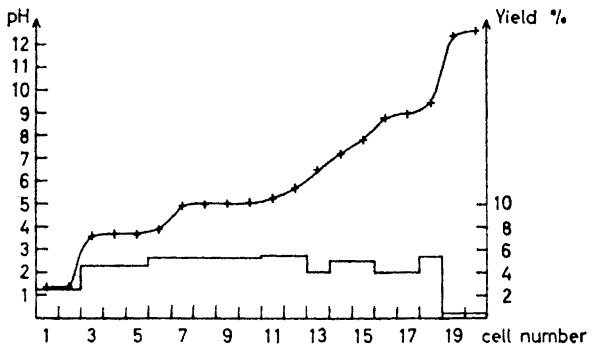

Fig. 3. Analysis of fractions from an isoelectric separation of the products of Experiment 1. pH at 20°C + − +, and yields in % of the total amount of ampholytes ———. The total yield of ampholytes was 22 g.

EXPERIMENTS AND RESULTS

Experiment 1. 0.13 mole of ethylene diamine, 0.2 mole of acrylic acid and 35 ml of water. The result of the electrofocusing is shown in Fig. 3. The pH range of the ampholytes extended from 3.8 to 9.5. The pH course showed two

plateaus at pH 3.8 and 5.0, respectively. Titration of samples from the fractions with pH 3.8 revealed that the fractions in this plateau contained ampholytes having three carboxylic groups added to the ethylene diamine molecule. These ampholytes were thus isoelectric in the pH range of dissociating carboxylic groups with closely spaced pK values giving the ampholytes a comparatively high buffering capacity of 2.2 mequiv./g·pH. For reasons of comparison, the buffering capacity of a known ampholyte, considered a good carrier ampholyte, may be calculated; using eqn. 11 in Ref. 2 a buffering capacity of 0.88 mequiv./g·pH is obtained for histidine.

The ampholytes isoelectric at pH 5.0 could be shown by titration to have two carboxylic groups, one of which with a pK at about 3.7. The lowest amino pK was localized at 6.8, thus a pI of 5.25 is calculated, to be compared with the pI 5.35 of the tetraprotic ampholyte of Table 3. The low pH value of the plateau (5.0) in this experiment may be due to the presence of isomers. Due to the comparatively big difference between the pK values on either side of the pI of the ampholyte, the buffering capacity was only 0.9 mequiv./g·pH.

The ampholytes focusing in the pH range 6.8 to 9.6 were by titration found to contain only one carboxylic group with a pK of about 3.7 and being isoelectric between the pK values of the two amino groups which were before coupling at 6.8 and 9.6 at 25°C. The inflexion point between the pK values on the titration curve was determined to 8.3 which should be the pI of this ampholyte. This is fairly close to the pI value 8.35 calculated from the pK of the amine. This also shows that the pK of the amines does not change much upon substitution with propionic acid. The buffering capacity was determined to 1.1 mequiv./g·pH. The yield of the different types of ampholytes was calculated from the dry weights in the different cells. About 4/16 of the ampholytes were found to contain three, 7/16 two, and 5/16 one carboxylic group. One may conclude from this that the addition of one propionic acid residue does not significantly facilitate or hinder the addition of more acidic groups.

Experiment 2. 0.15 mole of triethylene tetramine, 0.33 mole of acrylic acid and 35 ml of water. The result of the electrofocusing is shown in Fig. 4.

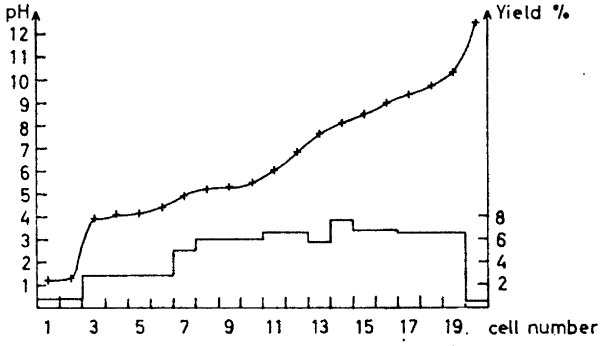

Fig. 4. Analysis of fractions from an isoelectric separation of the products of Experiment 2. For symbols see Fig. 2. The total yield of ampholytes was 45 g.

The resulting pH range of the ampholytes extended from 4 to 10. The pH course showed plateaus at 4.0 and 5.2, respectively. From the pK values of the amine it may be concluded that the fractions isoelectric around 4.0 consisted of ampholytes containing four carboxylic groups. The ampholytes isoelectric at pH 5.2 probably contained three carboxylic groups. The buffering capacity and conductivity of the fractions are given in Table 4. A rather even concentration was obtained in the pH range 3.8 to 9.5. In other experiments, where a higher ratio of acid to amine was used, the yield of ampholytes with pI below 7 increased markedly.

Table 4. Buffering capacity and conductance of fractions from Experiment 2.

pH of fraction	Buffering capacity mequiv./g·pH	$(m\Omega)^{-1}$·cm per mg in 10 ml
3.9	3.0	2.9
4.0—4.5	2.7	2.0
5.2	0.8	0.7
6.2	0.9	0.8
7.7	1.3	2.4
8.8	1.9	2.5
9.5	2.2	—

Experiment 3. 0.03 mole of pentaethylene hexamine with b.p. 180°—185°C at 0.05 mm Hg, 0.09 mole of acrylic acid, and 30 ml of water. The result of the electrofocusing is shown in Fig. 5. The pH range of the ampholytes extended from 3.5 to 9.5. A rather linear pH course was obtained, which was also to be expected with an amine having many pK values distributed in the above-mentioned pH interval. Determination of the buffering capacity in the fractions revealed a minimum of 1.1 mequiv./g·pH in the pH region between 5 and 8. This value was somewhat higher than in the corresponding interval of triethylene tetramine ampholytes, *cf.* Table 4. Higher values for pentaethylene

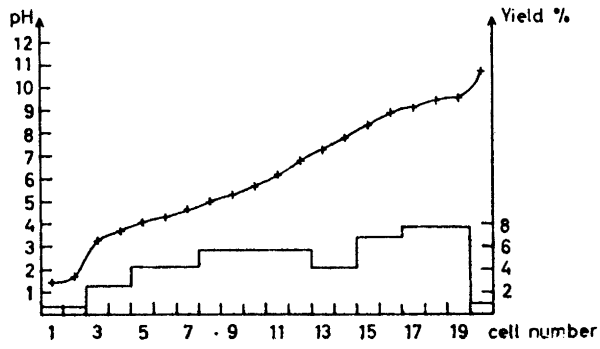

Fig. 5. Analysis of fractions from an isoelectric separation of the products of Experiment 3. For symbols see Fig. 2. The total yield of ampholytes was 15 g.

hexamine were also to be expected because the more amino groups are in the molecule, the more closely spaced are the pK values, as can be seen in Fig. 1.

Concerning the yields at different pH's, see Fig. 5. About as much ampholytes isoelectric above as below pH 7 was obtained.

Light absorption and solubility of the carrier ampholytes

It was found that the light absorption of the carrier ampholytes above 250 nm was mainly due to impurities in the amines. The commercial grade of purity gave a very high light absorption. When using distilled amines having a low absorbance, carrier ampholytes could be obtained with triethylene tetramine, which showed $E_{280}(1\ \%)$ values of about 0.1. It was, however, difficult to get such low values when using tetraethylene pentamine and pentaethylene hexamine. This problem was, however, overcome by making similar amines by a new synthesis procedure (O. Vesterberg. *To be published*).

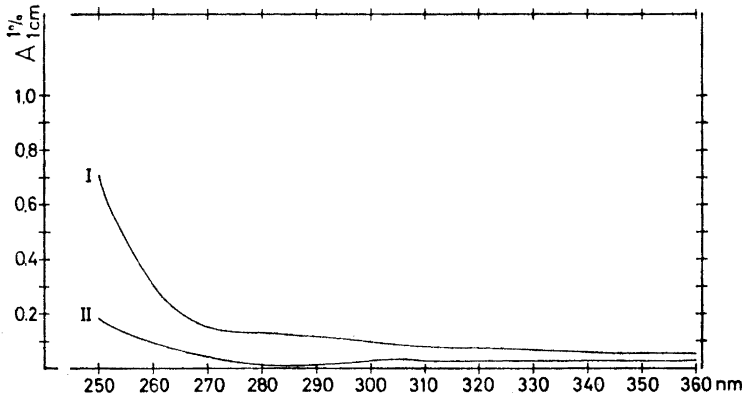

Fig. 6. Light absorbance of water solutions of isoelectrically focused and fractionated carrier ampholytes 1 % (w/v), using distilled water as a blank.
I, of a fraction with pH 6.9 of the type described in Experiment 2.
II, of a fraction with pH 7.0 of carrier ampholytes manufactured by LKB-Produkter AB.
The curves are representative for fractions with other pH values of each type of carrier ampholytes.

Similar carrier ampholytes are now made by LKB-Produkter AB, Stockholm. The manufacturer has improved the synthesis and the electrofocusing procedure, which has resulted into a still lower light absorption of the product. (Fig. 6.)

The carrier ampholytes have a very good solubility in water. They are now offered in a 40 % solution in water by LKB-Produkter AB.

The aliphatic polyamino-polycarboxylic type of carrier ampholytes are thus superior, both in respect to light absorption and solubility, to ampholytes containing an aromatic structure, which generally gives a high absorbance in ultraviolet, and a poor solubility in water.

DISCUSSION

The criteria which ampholytes must satisfy in order to be useful as carrier ampholytes have been treated by Svensson [2] and also in the first part of this paper. Because it was obvious that many substances with different pI's were needed, it was more tempting to find one synthesis method which could give many homologues with different pI's in one step, than to make different types of chemicals by various synthetic methods, although this meant that mixtures of different ampholytes were obtained.

Before the properties of the compounds synthesized can be discussed, we have to say something about the separation achievements. The shape of the pH courses gives an indication of the pI's, the relative amounts, and the buffering capacities of the electrolytes in the system. The addition of acrylic acid to the aliphatic amines was almost complete. This can be seen from results of electrofocusing, since remaining acid will be collected at the anode and remaining amine at the cathode. In Figs. 3, 4, and 5, the low pH at the anode and the high pH at the cathode are due to the added H_2SO_4 and NaOH, respectively. If unreacted acid and amine were present in appreciable amounts, many more cells would have got low and high pH's, respectively. Sulphuric acid and sodium hydroxide were used to prevent the ampholytes from contact with the electrodes. Experiments where H_2SO_4 and NaOH were excluded showed that the amounts of free acid and free amine were very small in the synthetic mixture. This means that the yield of ampholytes was close to 100 %. When electrolysing reaction mixtures not allowed to go to completion, many cells were occupied by acid and amine, and few cells were left for the ampholytes.

Concerning the choice of acid, other $\alpha-\beta$ unsaturated carboxylic acids than acrylic acid will also couple to the amines, but not as easily and completely as acrylic acid. Crotonic acid reacts easier than methacrylic acid. Any product containing large amounts of organic or inorganic acid or base is not well suited for isoelectric fractionation. In any case halogen ions must be excluded from the solution, because these ions will otherwise be converted to free halogens at the anode and by diffusion to cells containing ampholytes they may in turn oxydize them to yellow products. This makes coupling reactions with halogen substituted acids less suitable.

As was mentioned previously, it is possible to focus ampholytes at their respective pI's by electrolysis. It is, however, according to the theory, usually impossible to separate the carrier ampholytes completely by electrofocusing. The concentration distributions will look like overlapping, bell-shaped curves with maxima at the respective pI's. The degree of separation has been tested by renewed electrofocusing of the content of one cell in the electrolyzer. The interpretation of such experiments is that each cell contains a mixture of ampholytes with adjacent pI's. Experiments with coloured ampholytes have shown that most of such ampholytes will be focused to two or three cells, provided that there are many ampholytes with different pI's as in Experiment 3. If ampholytes with adjacent pI values have comparable buffering capacities, one ampholyte can only be assumed to dominate quantitatively at its own pI if the total amount of each neighbouring ampholyte is equal

or lower. The conclusion from the above is that it is generally more correct to speak about ampholytes isoelectric in a certain pH range, than to speak about separate ampholytes, if many ampholytes with closely spaced pI values are present. If the ampholytes are well focused and fractionated it is, however, valuable to determine the yields in different pH intervals, to titrate samples thereof and to measure conductivity. The absolute conductivity values are of less interest because they depend of the degree of focusing and separation of the ampholytes; the relative values are, however, interesting for comparison. Only if the pK values are not more closely spaced than two pH units it is possible to determine the pK's, provided that there is no greater difference in the corresponding pK values of isomers. The restrictions mentioned above means that all the values of pK's, buffering capacity, and conductivity given in this paper should be regarded with some caution. In the case of ethylene diamine the ampholytes obtainable are rather few, *cf.* Experiment 1. It is therefore possible to estimate pK and pI values from the titration curves. These values seem to agree rather well with both theoretical predictions and pI values determined by isoelectric focusing. Small discrepancies are, however, observed. It must be remembered that as soon as two or more propionic acid residues are added to the amines, isomers can be obtained, which probably have slightly different pK values. The conclusion from the experiments is that the pK of the amino groups is only shifted by very little upon substitution, and therefore the pI of the ampholytes is predictable to a high degree. Furthermore, the yields of different ampholytes depend on the type of amine and the proportion of acrylic acid used.

In Experiment 2 a minimum buffering capacity was observed for the ampholytes of the fractions taken at pH 5.2 and 6.2 (Table 4). Although the values may seem low, the buffering capacity is of the same order as for histidine which is considered a good carrier ampholyte. In accordance with the theory, the fractions having a minimum buffering capacity also showed the lowest conductivity. As was to be expected, ampholytes of pentaethylene hexamine showed good buffering capacity and conductivity. If using different amines with closely spaced pK values mixtures of isomers and homologues of ampholytes are obtained. With these it is possible to realize a good distribution of the conductivity.

When dealing with proteins with unknown pI's, or with proteins having widely different pI values, it is necessary to have many carrier ampholytes, the pI's of which cover the pH range 3 to 10. For more detailed studies of proteins at high resolution it is necessary to have carrier ampholytes giving a pH course essentially covering only the pI interval of the proteins under study, because the separability and the precision in the pI determinations depends on the shallowness of the pH course.[3] For this reason it is desirable to isolate the carrier ampholytes in groups having their pI's in ranges of one to two pH units. Experiments have shown that such fractionation can be well achieved in the multimembrane electrolyzer on a comparable degree of focusing as in the experiments reported here. Numerous applications on focusing and separation of various proteins with different pI values, made possible with these polyamino-polypropionic acid mixtures, have demonstrated the value

of this carrier ampholyte system.[3,9–16] * Patent on the synthesis and the fractionation of these carrier ampholytes is pending in several countries since 1964.

Acknowledgements. The author is greatly indebted to Professor H. Rilbe at the Department of Physical Chemistry, Chalmers Institute of Technology, Gothenburg, for valuable help and stimulating criticism and to Professor H. Theorell at the Biochemical Department of the Nobel Medical Institute, Stockholm, for interest in the project and for laboratory facilities. Thanks are also due to Mr. F. Forchheimer for collaboration in the construction of the electrolyzer.

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Received April 1, 1968.

* *Note added in proof.* For recent references, see Haglund, H. In Glick, D. *Methods in Biochemical Analysis*, Interscience, New York. *In press.*