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COMPARISON OF THE BUFFER CAPACITIES OF AMPHOLINE AND SERVALYT CARRIER AMPHOLYTE SYSTEMS USED IN ISOELECTRIC FOCUSING

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SUMMARY

The buffer properties of the Ampholine and Servalyt carrier ampholyte systems have been investigated by titration of (a) 2-ml fractions from pH gradients covering the pH ranges 3–10 and 5–7 and (b) the corresponding unfocused mixtures.

For most of the pH interval studied, the buffer capacity of an Ampholine pH gradient was found to be considerably higher than that of a Servalyt pH gradient with the same average concentration of carrier ampholytes (1%, w/v). Below pH 8 the Ampholines buffer 1.5–2.5 times better than the Servalyts, whereas above pH 8.5 the buffer capacities of the two systems were found to be equal, within experimental error. The minimum buffer capacity observed with the Servalyt system is very low, being only 1.8 μequiv./pH·ml at pH 6.2.

As would be expected, the buffer capacities relating to unfocused mixtures of carrier ampholytes were generally found to be much higher than those relating to the corresponding pH gradient. They are therefore of little use as a measure of the buffer properties of focused carrier ampholytes.

INTRODUCTION

For several reasons, the carrier ampholytes used for creating a natural pH gradient in the isoelectric focusing of, for instance, proteins should have buffer capacities that are as high as possible in and near their isoelectric states. As has been shown theoretically by Svensson[1,2], it is thus impossible to obtain a complete separation of two proteins isolectric on the same side of the (neutral) pH of the pure solvent unless the buffer capacities of the intermediary carrier ampholyte species are high enough to permit them to dictate the pH course in the steady state. Further, Svensson[2] has deduced that a high buffer capacity of an isoelectric carrier ampholyte also leads to a high conductivity, which is desirable, especially in the neutral pH region, in order to avoid local overheating due to rarefaction of ions.

A third argument for the use of carrier ampholytes with high buffer capacities has been presented recently[3]. Provided that the final pH gradient is dictated by the

carrier ampholytes, it was shown to be possible, in principle, to evaluate p*I* values of the sample species that are valid at the temperature of focusing, normally 4°, although the pH gradient is mapped at the more convenient temperature of 25°.

The carrier ampholyte system currently most widely used, namely Ampholine produced by Aminkemi (Stockholm, Sweden), is normally used at an average concentration of 1% (w/v). To judge from data presented by Davies[4] and Vesterberg[5], the buffer capacities of the focused Ampholine species at this concentration would be sufficient to permit them to dictate the pH gradient even in the presence of as much as 1% of protein. Fredriksson[3] demonstrated, however, that there may be several exceptions to this rule of thumb, especially among proteins isoelectric in the pH region 5–7, where the buffer capacity of the Ampholine is at a minimum. If it is assumed that a focused protein has a negligible effect on the pH gradient as long as its buffer capacity is less than 10% of that of the surrounding Ampholine molecules, it was evident from the data given that the protein concentration in the focused zone then has to be less than 0.5% for bovine serum albumin and less than 0.2% for bovine β-lactoglobulin A.

With this knowledge, it is of interest to note a brochure from Serva (Heidelberg, G.F.R.)[6] which presents data on their Servalyt carrier ampholyte system introduced some time ago. Whereas, according to Davies[4], the buffer capacity of an Ampholine pH gradient ranging from pH 3 to 10 is about 6 μequiv. per pH unit per millilitre of a 1% solution in the pH region 5–7, the corresponding buffer capacity of a Servalyt pH gradient ranging from pH 2–11 is claimed to be as much as 650 μequiv./pH·ml (see Fig. 10 in the brochure[6]).

At first sight, the Servalyt buffer capacity was impressive. However, on comparison of the data in Fig. 10 in the brochure[6] with the values printed on the bottles pictured on the front page of the brochure, it was evident that the buffer capacities given in the figure referred to a 40% solution rather than to a 1% solution as stated. In addition to this error, a closer study of the brochure also revealed that the buffer data of the Servalyt system has been evaluated from the titration curve of the *unfocused* mixture. Needless to say, this is an inadequate method which may give seriously misleading buffer capacities of the carrier ampholytes, as only a few of the many species in the unfocused mixture will be present at a given pH in the corresponding pH gradient.

As it is essential, in view of the above points, to use the carrier ampholyte system that has the best buffering properties in the isoelectric state, it was considered urgent to establish whether the focused Servalyt species also have buffer capacities that are constantly higher than the corresponding capacities of the Ampholine species, and an investigation of this aspect is described here.

EXPERIMENTAL

Isoelectric focusing

Solutions containing 1% (w/v) of Ampholine and Servalyt carrier ampholytes were focused simultaneously for 60 h at 4° in two LKB 110-ml columns. The temperature was then slowly increased to 25° over a period of 5 h and the focusing was continued at this temperature for a further 3 h. The anode was at the bottom of each column, 0.085 *M* sulphuric acid being used as the anode solution and 0.025 *M* sodium hydroxide solution as the cathode solution. The power was kept below 1 W

and the final voltage was 600 V. After the runs were finished, the column contents were fractionated into 2-ml fractions, which were stored in a freezer until required for titration.

Two pH ranges of each carrier ampholyte system were studied: for Ampholine pH 3.5–10 and 5–7, and for Servalyt pH 2–11 and 5–7. To test the repeatability, each of the pH gradients 5–7 were run in duplicate.

Evaluation of buffer capacity

By means of Radiometer automatic titration equipment (consisting of a Type TTT1 automatic titrator, Type SBU1 syringe burette unit, Type SBR2 syringe burette recorder and Type TTA31 titration assembly), titration curves for the fractions were recorded as follows. The pH electrode chain was standardized against a commercial buffer of appropriate acidity (Merck Pufferlösung, pH 3.00, 4.00, 5.00, etc.) and the pH value (pH_0) of a given fraction at 25° was measured. Then 0.050 ml of 0.5 *M* hydrochloric acid was added to an accurately measured part of the fraction (1.80 ml), whereupon the resulting solution was automatically titrated with 0.5 ml of 0.100 *M* sodium hydroxide solution under nitrogen. The syringe burette and the recorder had been calibrated in advance by titration of potassium hydrogen phthalate with 0.100 *M* sodium hydroxide solution.

In order to obtain the buffer capacity of the fraction in question at $pH = pH_0$, a third-order polynomial was fitted by means of a desk computer (Wang 700B) to a small part of the titration curve around pH_0. From the derivative at $pH = pH_0$, the desired buffer capacity (microequivalents per pH unit per millilitre of 1 % solution) could be calculated.

In this calculation, no correction was made for the dilution of the sample during the titration because the buffer capacity of the amount of water added before the point $pH = pH_0$ was reached (0.3 ml) was found to be negligible, at least in the pH interval 3–10, compared with the buffer capacities of the carrier ampholytes in the sample and the total experimental error (see below).

For comparison, unfocused 1 % solutions of Ampholines and Servalyts were also titrated in the same manner. From the resulting titration curves, the buffer capacities at the pH values of the fractions of the corresponding pH gradients were evaluated.

To investigate the repeatability of this technique for measurement of buffer capacity, a total of six portions of one 1 % solution of carrier ampholytes were titrated on three occasions.

RESULTS AND DISCUSSION

The buffer capacities obtained for the Ampholine pH gradient 3.5–10 and the Servalyt pH gradient 2–11 are plotted in Fig. 1 against the pH values of the fractions taken. Equivalent data for a pH gradient of 5–7 for each carrier ampholyte system are presented in Fig. 2. The latter figure also shows the buffer capacities of the corresponding unfocused mixtures.

Before the buffer data given in the figures are discussed, a few comments should be made about their validity. It was found that the difference between the two sets of buffer capacities obtained for each of the Ampholine and Servalyt pH

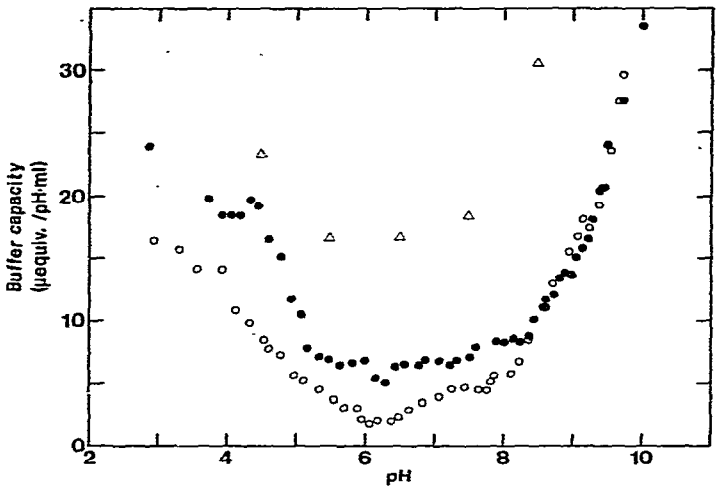

Fig. 1. Buffer capacities of a 1% Ampholine pH gradient of 3.5–10 (filled circles) and a 1% Servalyt pH gradient of 2–11 (open circles) obtained by titration of 2-ml fractions at 25°. The buffer capacity of each fraction is plotted against the pH value measured for this fraction before titration. The buffer capacities represented by triangles refer to an unfocused 1% solution of Servalyts of pH range 2–11 and were obtained by dividing the data given in Fig. 10 in ref. 6 by 40.

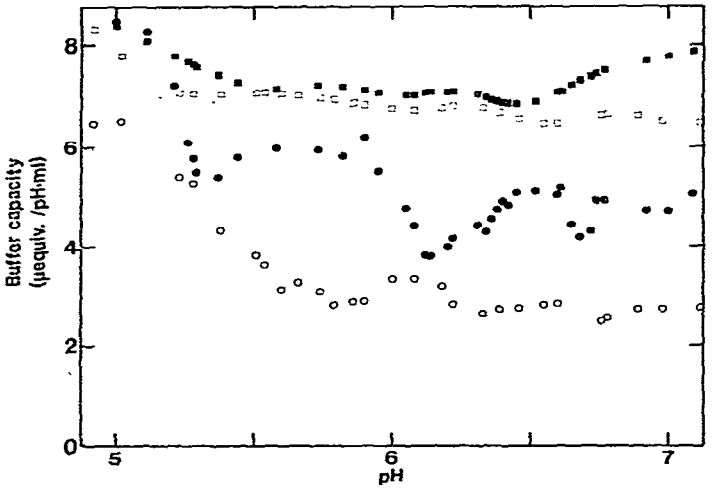

Fig. 2. Buffer capacities at 25° of 1% Ampholine of pH range 5–7 before (filled squares) and after (filled circles) isoelectric focusing, and of 1% Servalyt of pH range 5–7 before (open squares) and after (open circles) isoelectric focusing.

gradients 5–7 was 5–10% for most of the pH interval and 15% at some pH values. These differences are significantly higher than would be expected from errors in the recording and evaluation of the titration curves. Thus the buffer capacities determined for the six portions of a fixed carrier ampholyte solution showed a maximum divergency of only 2%. It should also be mentioned that the pH values measured for the portions before titration were constant to within $\pm$ 0.02 pH unit.

The comparatively large uncertainty in the buffer capacities of focused carrier ampholyte species seems reasonable, however, if one remembers that, according to theory[1], the concentration distributions of the ampholytes in the finished pH gradient will have the form of overlapping, bell-shaped curves with maxima at the p*I* values of the species. Consequently, the buffer capacities measured for fractions from a pH gradient will depend on the degree of focusing and separation of the ampholytes in the actual run as well as on the pH intervals covered by these fractions.

The data in Figs. 1 and 2 have not been corrected for the buffer capacities of the water and the sucrose in the fractions for two reasons. Firstly, it is the total buffer capacity of the pH gradient at a given pH that is of interest in the discussion of its resolving power, conductivity and temperature dependence. Secondly, the buffer capacities of the water and the sucrose are small in comparison with those of the ampholytes for most of the pH range studied and will therefore be of little significance in a comparison of the two carrier ampholyte systems.

Notwithstanding what has been said above concerning the uncertainty of the buffer data presented, they will undoubtedly be relevant for the purpose of comparing the buffer properties of the Ampholine and Servalyt carrier ampholyte systems, firstly because the compared pH gradients of the two systems were created under as identical conditions as possible, and secondly because for most of the pH range of interest, the differences in the buffer capacities of the Ampholines and the Servalyts far exceeds the experimental error.

As is evident from Fig. 1, the buffer data reported by Serva[6] would be seriously misleading if regarded as a measure of the buffer capacity of a finished pH gradient of 2–11, even if one considered (as has been done in Fig. 1) that they probably refer to an average carrier ampholyte concentration of 40% instead of 1% as stated. In the pH region 6–7, for example, the buffer capacity of a 1% solution should be about 16 μequiv./pH·ml according to Serva, whereas the present investigation gives a value 2–3 μequiv./pH·ml.

Apart from the buffer data of the Servalyt system given in the Serva brochure[6], the label of each Servalyt bottle states the buffer capacity of the contents in the pertinent pH range. For the bottle of Servalyt of pH gradient 2–11 used to obtain the open circles in Fig. 1, the buffer capacity stated was 1720 μequiv. per millilitre of 40% solution. To judge from the data in Fig. 1, this type of information is inadequate and of little use and should thus be omitted.

A further study of Fig. 1 reveals that below pH 8, the buffer capacity of a Servalyt pH gradient of 2–11 is 1.5–2.5 times lower than that of an Ampholine pH gradient of 3.5–10, whereas for pH values above 8.5, the buffer capacities of the two gradients are equal, within experimental error. For both gradients, the buffer capacity is at a minimum around pH 6.2. The minimum value for the Ampholine gradient is 5.2 μequiv./pH·ml, which is in good agreement with the value of 5.7 determined by Davies in 4-ml fractions[4]. The minimum buffer capacity of the Servalyt pH gradient of 2–11 is very low, being only 1.8 μequiv./pH·ml.

If the buffer data of the pH gradients 5–7 are compared (Fig. 2), it can be seen that the Ampholine gradient has the higher buffer capacity throughout; for most of the pH interval 5–7, this gradient buffers 50–75% better than the Servalyt gradient. Yet the buffer properties of the Ampholine pH gradient of 5–7 are unsatisfactory in certain respects. As is evident from Fig. 2, deep buffer minima of 3.8 and 4.2 μequiv./

pH·ml were obtained at pH 6.2 and 6.7, respectively*. Of course, the presence of such pronounced minima in the buffer capacity is undesirable in itself. It also implies, however, that pronounced minima in the conductivity distribution of the Ampholine pH gradient of 5–7 are to be expected[2]**. Besides giving rise to an uneven distribution of the field strength along the gradient with an accompanying uneven effect of focusing, the conductivity minima may cause local overheating, which, in turn, induces convectional breakdown of a density gradient or drying out and deformation of a gel plate.

In conclusion, this investigation has confirmed the supposition that buffer data relating to unfocused carrier ampholyte mixtures are of little use as a measure of the buffer properties of focused carrier ampholytes. The investigation has also clearly shown that the buffer properties of focused Ampholines are superior to those of focused Servalyts for most of the pH range of interest. However, it would be desirable in the future to improve the Ampholine system further by adding "tailor-made" species isoelectric around pH 6.2 and 6.7 and with good buffering properties.

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* In view of the data in Fig. 1, the minimum values are unexpectedly low. They were not a result of accidental experimental errors, however, but were closely repeated in the duplicate run.

** In fact, the positions of the buffer minima in Fig. 2 agree well with those found by Davies[4] in the conductivity course of a pH gradient of 5–8 if one considers that his pH measurements were made at 4°.