Journal of Chromatography, 209 (1981) 265–272
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,617

MOLECULAR WEIGHT DISTRIBUTION OF CARRIER AMPHOLYTES FOR ISOELECTRIC FOCUSING

ADRIANA BIANCHI BOSISIO
Clinical Chemistry Laboratory, S. Carlo Borromeo Hospital, Via Pio Secondo 3, Milan 20153 (Italy)
ROBERT S. SNYDER
Marshall Space Flight Center, NASA, ES-73, Huntsville, AL 35812 (U.S.A.)
and
PIER GIORGIO RIGHETTI*
Department of Biochemistry, University of Milan, Via Celoria 2, Milan 20133 (Italy)
(Received January 3rd, 1981)

SUMMARY

By osmolarity, gel filtration and dialysis experiments it has been demonstrated that Ampholine carrier ampholytes have relative molecular masses (M_r) ranging from 600 daltons in the alkaline range (pH 8–10) up to 900 daltons in the acidic range (pH 3.5–5), with graded intervals for the intermediate ranges. The most acidic range (pH 2.5–4) has a considerably smaller $\bar{M}_r$ (325 daltons), suggesting that it is synthesized with different polyamines and/or acids. There is no indication that Ampholines with $\bar{M}_r > 1000$ daltons exist. Evidence is presented that carrier ampholytes aggregate in solution, giving rise to complexes with higher apparent $\bar{M}_r$.

INTRODUCTION

Considerable interest has recently been focused on the relative molecular mass (M_r) of carrier ampholytes (Ampholine, Servalyte and Pharmalyte) used in isoelectric focusing (IEF). This property plays a fundamental role in the IEF process itself, both in the kinetics of pH gradient formation and in the subsequent separation of the fractionated proteins from the ampholyte buffers. Clearly, a low M_r for the latter species is highly desirable, and the original synthetic process of Vesterberg[1] was thought to satisfy this requirement. The general, physico-chemical properties of carrier ampholytes have been described by Haglund[2], Vesterberg[3] and Righetti and co-workers[4,5]. Direct measurements, made by gel filtration, had given a $\bar{M}_r = 700$ for Ampholine[2], with only 0.7% of the species above 1000 daltons and negligible amounts (*ca.* 0.03%) in the proximity of 4000 daltons[6]. Gasparic and Rosengren[7] had also succeeded in synthesizing lower M_r species (300 daltons) for IEF separations of peptides. These data, which come from the manufacturers, might be regarded with suspicion by the scientific community, but indeed have been fully substantiated by the independent work of Gelsema's group[8] who, on the basis of specific conductivities

0021-9673/81/0000–0000/$02.50

and buffering capacities of carrier ampholytes, calculated a $\bar{M}_r$ = 710 for Ampholine and $\bar{M}_r$ = 790 for Pharmalyte.

On the other hand, these $\bar{M}_r$ values have recently been questioned by several research groups, who have proposed all possible ranges of $\bar{M}_r$s: 5000–7000 daltons[9]; 800–1200 daltons for Servalyte, 1000–6000 daltons for Ampholine and 1000–15,000 daltons for Pharmalyte[10,11]; even an upper limit of 20,000 daltons has been suggested[12]. These high $\bar{M}_r$ values apparently are not just exhibited by a tiny fraction of the population of carrier ampholytes, but by a substantial proportion of them[10,11], especially at alkaline pH[9].

These highly conflicting data prompted an investigation by our group on the real $\bar{M}_r$ of Ampholine, and the results are reported in this paper.

EXPERIMENTAL

Spermine [N,N′-bis(3-aminopropyl)-1,4 butanediamine] free base (M_r = 202) was obtained from Sigma (St. Louis, MO, U.S.A.), and tetraethylenepentamine (TEPA) (M_r = 186) and pentaethylenehexamine (PEHA) (M_r = 229) from Hoechst Italia (Milan, Italy). Cytochrome *c* (Cyt C; equine heart; M_r = 12,400) and myoglobin (Myo; whale skeletal muscle; M_r = 17,800) were obtained from Calbiochem (La Jolla, CA, U.S.A.). Several batches of Ampholine carrier ampholytes, manufactured during 1972–80 by LKB (Bromma, Sweden) were tested. All the narrow pH ranges and also the wide pH 3.5–10 range were analysed, except for the pH range 9–11, which was not available at the time of this study.

Gel permeation studies

For molecular mass determination, we used a Pharmacia (Uppsala, Sweden) thin-layer chamber. Gel chromatography was performed on 20 × 20 cm glass plates coated with a 1-mm layer of Bio-Gel P-10 (Bio-Rad Labs., Richmond, CA, U.S.A.) in 25 m*M* phosphate buffer (pH 7.25). All the M_r markers were dissolved in the same buffer, at a concentration of 0.5% for spermine, Cyt C and Myo and 1% for TEPA, PEHA and all the Ampholine pH ranges. A 10-μl volume of each solution was spotted on the plate. The gel filtration was allowed to proceed for *ca.* 3 h at room temperature, then a print was taken with a Whatman 3MM paper for 30 sec. The paper was dried in an oven at 110°C for 5 min and then sprayed with ninhydrin reagent. The violet colour of the spot appeared after incubation in the oven at 100°C for 20 min. Myo and Cyt C were visible on the paper print as brownish spots due to Fe^{3+}.

Osmolarity measurements

These were made with an Osmette A Automatic Oxmometer from Precision Systems (Boston, MA, U.S.A.). The following Ampholine pH ranges were tested: pH 2.5–4 (batch 12, Dec. 1976); pH 3.5–5 (batch 15, Aug. 1976); pH 4–6 (batch 30, Oct. 1976); pH 5–7 (batch 27, Jan. 1977); pH 6–8 (batch 2, April 1973); pH 7–9 (batch 47, Sept. 1972); pH 8–10 (batch 40, March 1972); and pH 3.5–10 (batch 67, March 1980). All batches were diluted with distilled water to a final concentration of 1% (w/v) and tested as such. More dilute solutions (0.5%, w/v) were also used, but they gave very low osmolarity readings with a greater spread of values.

In order to assess the reproducibility of the data and of the osmometer, ten

different dilutions of each pH range were prepared and measured over a period of 1 week. Each sample was read three times, so that each point of the curve in Fig. 3 represents an average of 30 measurements.

Dialysis experiments

Dialysis studies were performed with Spectrapor membranes (having a nominal molecular mass cut-off of 3500 daltons) from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Volumes of 0.5 ml of 10% Ampholine solutions were dialysed against 100 ml of distilled water and 100 m*M*, 500 m*M* and 1 *M* sodium chloride solutions at room temperature, for periods up to 24 h, with no changes to the dialysis solution. When dialysed against salt, the Ampholine solutions contained the same sodium chloride molarity as the dialysis liquid. Aliquots of the dialysate were assayed for Ampholine content, at different time intervals, by the nihydrin assay.

RESULTS

Fig. 1 shows the results of a 24-h dialysis of Ampholine pH 3.5–10 in a Spectrapor bag with a nominal cut-off of 3500 daltons. In the control, after a 24-h period,

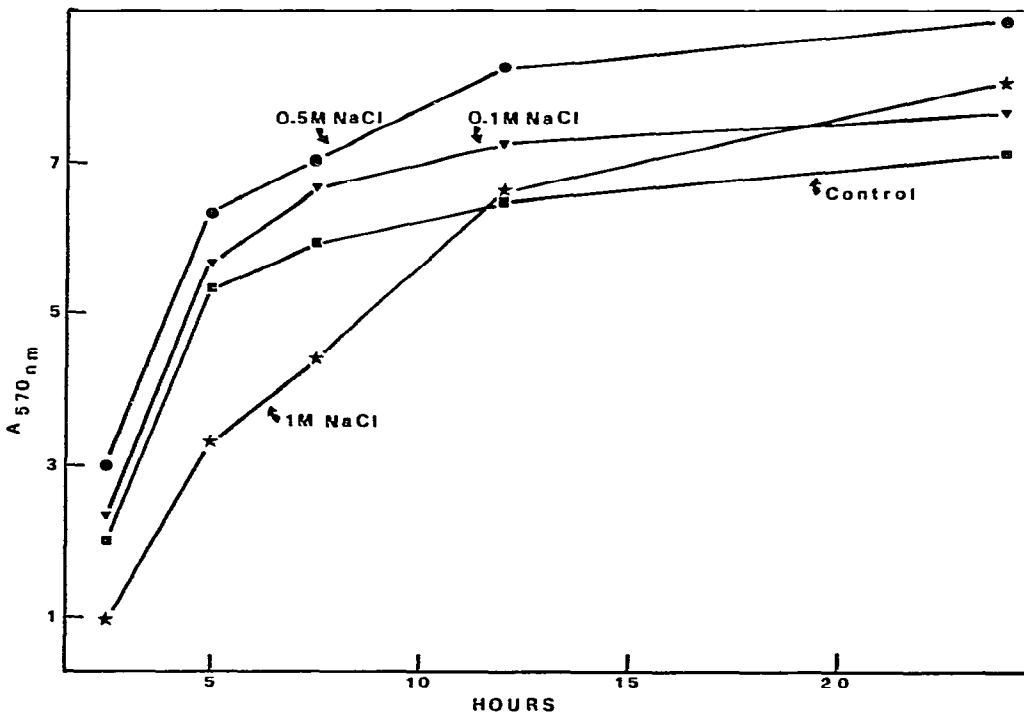
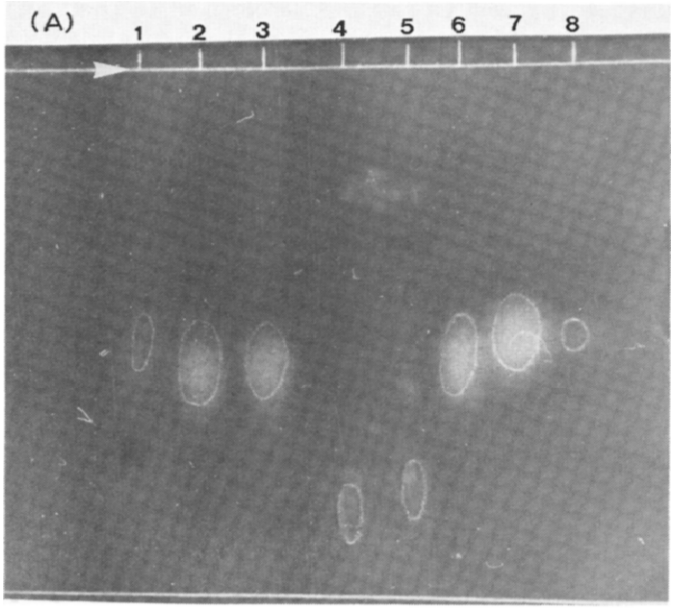


Fig. 1. 24-h dialysis of carrier ampholytes: 0.5 ml of 10% Ampholine pH 3.5–10 was dialysed against 100 ml of water (control) or 0.1, 0.5 and 1 *M* NaCl in a Spectrapor membrane with an $\bar{M}_r$ cut-off of 3500 daltons. At the given time intervals, 1-ml aliquots (in triplicate) of the dialysate were assayed for Ampholine by ninhydrin staining. In the NaCl experiments, salt was also added to the solution used to prepare the colorimetric calibration graph, as it was found to interfere with the ninhydrin colour development.



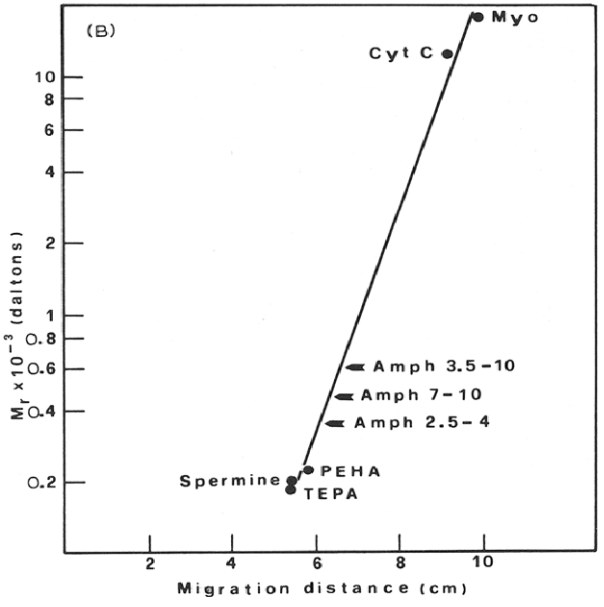


Fig. 2. (A) Paper print after gel filtration on a thin layer of Bio-Gel P-10. Spots: 1 = Ampholine pH 2.5–4; 2 = Ampholine pH 3.5–10; 3 = Ampholine pH 7–10; 4 = myoglobin; 5 = cytochrome *c*; 6 = PEHA; 7 = TEPA; 8 = spermine. The arrow indicates the origin. (B) Semi-logarithmic plot of the gel filtration data in A.

about 70% of the Ampholine had been leached out in the dialysate. Clearly, if Ampholine had the high M_r values reported[9,10], they would have hardly passed the membrane (under these conditions insulin, with M_r = 6000, was effectively retained by the Spectrapor envelope). Interestingly, 0.1 *M* and, to a much greater extent, 0.5 *M* sodium chloride considerably accelerated the dialysis process. In the latter instance, after 24 h, more than 85% of the contents of the bag had been emptied into the surrounding medium. This behaviour is consistent with a previous hypothesis by Gianazza *et al.*[13] that carrier ampholytes associate among themselves in solution. This hypothesis has recently been fully corroborated by the independent work of Gelsema *et al.*[14]. However, it cannot be excluded that other mechanisms might also be operating at the membrane level, as 1 *M* sodium chloride markedly inhibits the dialysis process, at least during the first half of the experimental period.

As a more direct approach, we tried thin-layer gel chromatography of different Ampholine pH ranges, using as M_r markers polyamines, which should more closely mimic the chromatographic behaviour of the carrier ampholytes. Fig. 2A shows the paper print, after gel filtration, stained with a ninhydrin spray. In Fig. 2B, we have attempted an M_r determination by the usual log M_r *vs.* migration plot. The "apparent" M_r values found were as follows: Ampholine pH 2.5–4, *ca.* 350 daltons; Ampholine pH 7–10, *ca.* 500 daltons; and Ampholine pH 3.5–10, *ca.* 600 daltons.

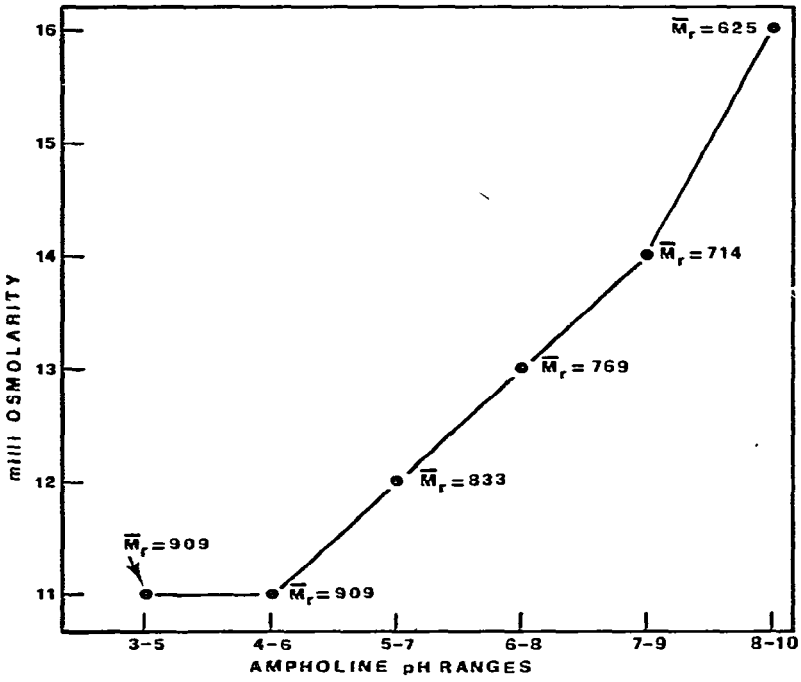


Fig. 3. Osmolarity measurements on narrow Ampholine pH ranges: 1% Ampholine solutions in the pH ranges 3.5–5, 4–6, 5–7, 6–8, 7–9 and 8–10 were read with an Osmette A Automatic Osmometer. Ten different dilutions of each pH range were prepared and read three times by different operators on different days. For each osmolarity value in each Ampholine pH range a corresponding average molecular mass ($\bar{M}_r$) was calculated. Ampholine pH 2.5–4 gave 31 ± 2 mOsm corresponding to $\bar{M}_r$ = 325.

These data must be regarded as only approximate, owing to a lack of appropriate M_r markers in the Ampholine region. Moreover, the fact that we can draw a straight line between the very low M_r polyamine and the relatively high M_r proteins could be purely accidental, as we have no intermediate M_r markers to map the course of this line. Nevertheless, these data demonstrate that the Ampholine buffers are clearly clustered around the low M_r polyamines and far removed from the Cyt C and Myo markers.

For small molecules, osmolarity measurements by the freezing-point depression method should still be the best approach to M_r determination, provided that they do not dissociate or associate. Therefore, 1% Ampholine solutions, in all the narrow ranges available, were subjected to osmolarity determinations, on the assumption that at this high dilution they would approach ideal behaviour in solution. For easy assimilation of the data, the values obtained are displayed graphically in Fig. 3, even though the abscissa does not really represent a physical function. For each milliosmole measurement, in each narrow pH range, an average value of molecular mass was calculated. It can be seen that, as is predictable from the Vesterberg synthesis[1,4,13], the alkaline pH ranges have the lowest and the acidic pH ranges the highest M_r. The molecular masses of the Ampholine buffers seem to go from a minimum of 600 up to a maximum of 900 daltons. However, when the most acidic pH range (pH 2.5–4) was analysed, it gave a milliosmole reading of 31 $\pm$ 2, which would correspond to $\bar{M}_r$ = 325 daltons, clearly far removed from the trend shown in Fig. 3.

DISCUSSION

Dialysis experiments

The results clearly indicate that Ampholine molecules associate with each other, as previously suggested by us[1,13] and as demonstrated recently by Gelsema *et al.*[14]. The fact that salts split these complexes suggests that they are prevalently ionically bonded. We believe this is so, especially between acidic and basic species, because they have complementary structures and the same distance between positive and negative charges, allowing for a very good "fit". If these aggregates do exist, another way to reduce them would be to dilute progressively the Ampholine solution, and in fact it has been demonstrated[14] that, in going from a 4% to a 0.6% Ampholine solution, these higher M_r complexes are concomitantly reduced, if not fully eliminated. This could explain the ultrafiltration data of Goerth and Radola[11], who have found retentions of Ampholine of as much as 90% in membranes with M_r cut-offs of 2000–4000 daltons. Because, during the ultrafiltration process, the initial solution of 2% Ampholine (in which aggregates already exist) is reduced by a factor of 10, if water is expelled from the pores at a faster rate than carrier ampholytes, this results in a progressive concentration of the Ampholine solution inside the bag, which automatically drives the equilibrium towards more aggregates, and higher $\bar{M}_r$ complexes, which could well have an average diameter greater than the membrane pore size. This could also explain the dialysis data of Gierthy *et al.*[9], who dialysed against distilled water alkaline carrier ampholytes in concentration ranges spanning more than 100-fold dilutions. The dialysis kinetics of the progressively more concentrated Ampholine solutions are clearly indicative of association phenomena (see Fig. 6 in ref. 9).

Osmolarity data

Surprisingly, no data on M_r determination of carrier ampholytes by this technique have been reported. However, provided there are no association–dissociation phenomena occurring among the solute molecules, this should be the best and most reliable method for M_r assessment. It should be noted that, in fact, all other methods, such as dialysis, gel filtration and gel electrophoresis, are indirect means for M_r measurements, based on certain assumptions about the physico-chemical behaviour of the solute molecules, behaviour which in turn is linked to their molecular mass. Clearly, if other, unwanted and unaccounted for, phenomena occur concomitantly, *e.g.*, adsorption by the dialysis membrane or by the gel filtration matrix, the M_r data will then be meaningless. In the case of SDS electrophoresis, for instance, the error could be very large. Hence, by this latter technique, the M_r of Ampholine pH 9–11 has been determined[9] to be 5000–7000 daltons, whereas a correct determination gives $\bar{M}_r \approx 400$ daltons[14]. There is no indication that SDS would bind to small basic molecules, whereas it binds poorly to basic proteins[15]; therefore the "retarded" electrophoretic mobility of basic Ampholine is probably due simply to a very low negative charge density.

Our data on osmolarity represent a compromise between a concentration of Ampholine low enough to minimize association phenomena yet high enough to allow for accurate readings with the Osmette apparatus. Therefore, a 1 % Ampholine solution was chosen as it allows readings above 10 mOsm where the scatter of results becomes acceptable. A series of readings at 0.5 % concentration gave the expected 0.5 mOsm values, but the reproducibility was poor. In any event, it should be noted that if some associated species were present in 1 % Ampholine, then our reported $\bar{M}_r$ values would be slightly overestimated, not underestimated. We also believe that our osmolarity data represent a safe procedure, as they were determined only in narrow pH ranges, *i.e.*, under conditions where they are quasi-isoelectric and therefore with minimal, if any, interactions[16]. Our data also demonstrate that the six pH ranges from pH 3.5–5 to 8–10 belong to the same "Vesterberg" family, *i.e.*, they are prepared in a single synthetic process and then subfractionated in multi-compartment electrolysers into narrow pH ranges. This automatically means that the alkaline species have smaller $\bar{M}_r$ than the acidic components (see Fig. 3). The fact that pH 2.5–4 Ampholine has such a small $\bar{M}_r$ (325) suggests that it does not belong to the "Vesterberg" family, *i.e.*, it is synthesized independently in a single step with other polyamines and/or acids (see also the discussion by Gelsema *et al.*[14]).

In conclusion, our data indicate that Ampholines (except for the low M_r pH 2.5–4 and pH 9–11 ranges) have $\bar{M}_r = 750 \pm 150$ daltons, and are distributed from a minimum of $\bar{M}_r = 600$ daltons in the alkaline (pH 8–10) range up to a maximum of $\bar{M}_r = 900$ daltons in the acidic (pH 3.5–5) range. There is no indication that Ampholine species with $\bar{M}_r \geqslant 1000$ daltons exist. Our data are corroborated by the following: (a) direct osmolarity measurements; (b) direct gel filtration measurements at high salt concentrations[14]; (c) theoretical considerations based on conductivity and buffering capacity[8]; (d) impossibility of fixing and staining in a polyacrylamide gel the carrier ampholytes by the "fast staining" procedure, which is known to fix and stain any peptide longer than 12–14 amino acids ($\bar{M}_r \geqslant 1500$ daltons)[17]; (e) impossibility of precipitating the carrier ampholytes with concentrations of ammonium sulphate up to 100 % saturation[18].

ACKNOWLEDGEMENTS

P.G.R. and A.B.B. thank the University Space Research Association (USRA) for a summer visiting professorship at the Marshall Space Flight Center (MSFC) and NASA for providing generous space and research facilities at MSFC.

REFERENCES

1 O. Vesterberg, *Acta Chem. Scand.*, 23 (1969) 2653–2666.
2 H. Haglund, in J. P. Arbuthnott and J. A. Beeley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, pp. 3–22.
3 O. Vesterberg, in N. Catsimpoolas (Editor), *Isoelectric Focusing*, Academic Press, New York, 1976, pp. 53–76.
4 P. G. Righetti, E. Gianazza and A. Bianchi Bosisio, in A. Frigerio and M. McCamish (Editors), *Recent Developments in Chromatography and Electrophoresis, 10*, Elsevier, Amsterdam, 1980, pp. 89–117.
5 P. G. Righetti, E. Gianazza and K. Ek, *J. Chromatogr.*, 184 (1980) 415–456.
6 V. Gasparic and A. Rosengren, in J. P. Arbuthnott and J. A. Beeley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, pp. 178–181.
7 V. Gasparic and A. Rosengren, *Sci. Tools*, 21 (1974) 1–2.
8 W. J. Gelsema, C. L. de Ligny and N. G. van der Veen, *J. Chromatogr.*, 173 (1979) 33–41.
9 J. F. Gierthy, K. A. O. Ellem and J. R. Kongsvik, *Anal. Biochem.*, 98 (1979) 27–35.
10 B. J. Radola, *Electrophoresis*, 1 (1980) 43–56.
11 K. Goerth and B. J. Radola, in B. J. Radola (Editor), *Electrophoresis '79*, Walter De Gruyter, Berlin, 1980, pp. 555–563.
12 G. Baumann and A. Chrambach, *Anal. Biochem.*, 64 (1975) 530–536.
13 E. Gianazza, C. Astorri and P. G. Righetti, *J. Chromatogr.*, 171 (1979) 161–170.
14 W. J. Gelsema, C. L. de Ligny and W. M. Blanken, *J. Chromatogr.*, 198 (1980) 301–316.
15 J. F. Poduslo and D. Rodbard, *Anal. Biochem.*, 101 (1980) 394–406.
16 H. Rilbe, *Ann. N.Y. Acad. Sci.*, 209 (1973) 11–22.
17 P. G. Righetti and F. Chillemi, *J. Chromatogr.*, 157 (1978) 243–251.
18 P. Nilsson, T. Wadström and O. Vesterberg, *Biochim. Biophys. Acta*, 221 (1970) 146–148.