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BEHAVIOR OF ANGIOTENSIN OCTAPEPTIDES AND DINITROPHENYL-ANGIOTENSIN DURING CHROMATOGRAPHY ON POLYACRYLAMIDE AND DEXTRAN GELS

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SUMMARY

The behavior of the synthetic angiotensin II octapeptides (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, molecular weight 1046, and its Asn[1]-Val[5] congener, molecular weight 1031) have been studied during gel chromatography on cross-linked polyacrylamide (Bio-Gel, P-2), and dextran (Sephadex G-15). In the case of the asparagine peptide adsorption was observed with both media. On polyacrylamide adsorption was marked at acid pH (0.05 *N* HCl), but at pH 5.5 in 0.1 *M* pyridinium acetate the asparagine compound could be separated from and emerged between a larger peptide (bacitracin, molecular weight 1411) and smaller molecules (pyridine–Cu^{2+} complex; Na^+). On dextran gel asparagine[1]-angiotensin was retarded due to adsorption not only at acid pH but also in 0.1 *M* pyridinium acetate, with adsorption even more marked in dilute alkali (0.01 *N* NH_4OH). Under none of these conditions was it possible with ordinary dextran gel to separate asparagine[1]-angiotensin from amino acids or salts. In sharp contrast, the aspartic acid[1]-angiotensin was excluded from G-15 at alkaline pH, and the two angiotensins were thus readily separated from one another under these conditions. Adsorption of the asparagine[1]-angiotensin was nearly abolished by 1 *M* pyridine. The separation of the two angiotensins under these conditions is attributed to the greater negative charge of the aspartic acid[1]-angiotensin which results in exclusion from the gel and overcomes the "aromatic" adsorption of the remainder of the molecule.

The dinitrophenyl (DNP) derivative of asparagine[1]-angiotensin is readily separable from DNP-amino acids by chromatography in absolute methanol through an alkylated dextran gel (Sephadex LH-20).

INTRODUCTION

During the course of the development of a double isotope derivative assay for the quantitation of angiotensin and other peptides as their ^3H-labeled dinitrophenyl (DNP) derivatives[1], we were interested in the possibility of separating, on the basis

of molecular size, the angiotensins (mol. wt. 1000–1250) from other larger peptides and from amino acids. Both of the latter groups of compounds would be present in biological extracts and form DNP derivatives which might interfere in the isotope derivative assay procedure as it has been devised, even though the DNP-angiotensin is extensively purified by thin-layer chromatography. The behavior of the angiotensins and of DNP-angiotensin during gel chromatography was therefore examined. This report presents previously undescribed adsorption effects which were observed with angiotensin octapeptides on both polyacrylamide and dextran gels.

METHODS AND MATERIALS

Gels

Polyacrylamide gel was obtained from Bio-Rad, Richmond, Calif., as Bio-Gel P-2, 50–100 mesh. Dextran gels were Sephadex G-15 (40–120 μ) and LH-20 (25–100 μ) from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Both P-2 and G-15 have exclusion limits of about 1500 for uncharged molecules. The gels were not prewashed or pretreated with any solvents other than those with which they were equilibrated and in which the chromatography was performed, except where indicated below.

Chemical compounds

Angiotensin II (aspartic acid^{1}-isoleucine^{5}) octapeptide and its ^{14}C analog labeled in the isoleucine^{5} position (specific activity, 215 mCi/mmole) were obtained from Schwarz Bio-Research, Orangeburg, N.Y. Angiotensin II amide (asparagine^{1}-valine^{5}-angiotensin octapeptide) [Hypertensin®] and aspartic acid^{1}-valine^{5}-angiotensin octapeptide were gifts of the CIBA Pharmaceutical Company, Summit, N.J., and CIBA, Ltd., Basle, Switzerland. Bacitracin (mol. wt. 1411) and DNP-amino acids were obtained from Mann Research Laboratories, New York. The stable and ^{14}C-labeled tri-DNP derivative of asparagine^{1}-angiotensin and its monomethyl ester were prepared in our laboratory as described elsewhere[1]. [^{14}C]Aspartic acid and [^{14}C]asparagine were obtained from New England Nuclear Corp., Boston, Mass.

Columns

The columns contained a bed of gel 1.5 cm × 30 cm or 0.9 cm × 68 cm as indicated. Compounds were applied in ±0.5 ml of the same solvent that was used for preparation and elution of the gel. Flow rates and fraction sizes are indicated in the legends.

Detection of compounds

For quantitation of μmole amounts of angiotensin, bacitracin, and histidine, 1-ml aliquots were assayed by the colorimetric method of ABRAHAM *et al.*[2] which uses the stabilized diazonium salt Fast Red TRN. DNP compounds and copper (or its pyridine complex) were determined by their optical densities at 340 and 600 nm, respectively. Microgram and nanogram quantities of unlabeled angiotensin were measured by the double isotope derivative method[1]. The [^{14}C]aspartic acid and [^{14}C]-asparagine were determined by liquid scintillation counting in dioxane. In the case of the latter two compounds stable carrier amounts of amino acid (1 μmole) were used during the chromatography on the long G-15 column. Sodium was detected qualitatively using a flame.

RESULTS

Polyacrylamide gel

A mixture of angiotensin and several marker compounds was passed through the P-2 column. With 0.05 *N* HCl as eluant (Fig. 1) separation of bacitracin (mol. wt. 1411), histidine (mol. wt. 155), and an inorganic ion (Cu^{2+}) was possible, although some overlap was evident. However, angiotensin amide was clearly retarded beyond expectation and emerged later than histidine. DNP-aspartic acid was grossly retarded (Fig. 1). Inclusion of 1 *M* NaCl with the HCl did not alter the elution positions. With 1 *M* NaCl (in the absence of acid) angiotensin emerged much earlier (peak at 44 ml) while copper, interestingly, was retarded (peak at 78 ml *versus* 58 ml in dilute acid).

At a pH of 5.5 in 0.1 *M* pyridinium acetate (Fig. 2) asparagine[1]-angiotensin was perhaps somewhat retarded beyond that expected from its size, but emerged between bacitracin (mol. wt. 1411) and the pyridine–Cu^{2+} complex (mol. wt. 380) and well before the Na^+ peak. (Under these conditions Cu^{2+} forms an intense blue pyridine complex containing four pyridine molecules per copper[2a]).

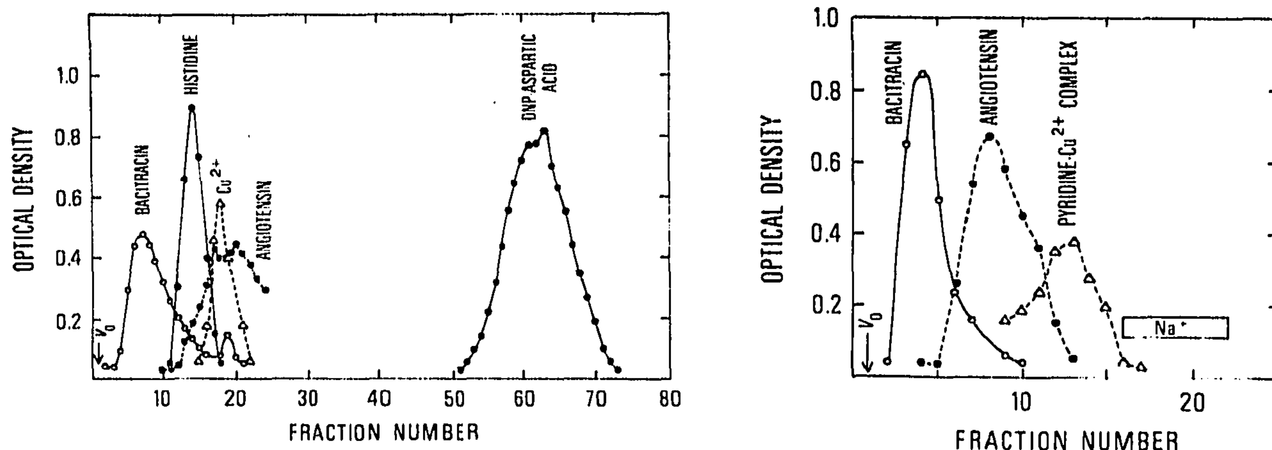


Fig. 1. Chromatography of 1 µmole each of asparagine[1]-angiotensin II, bacitracin, histidine, cupric nitrate, and DNP-aspartic acid on polyacrylamide gel (Bio-Gel, P-2). Note the position of angiotensin relative to histidine and copper. Eluting solvent, 0.05 *N* HCl; 2-ml fractions; flow rate, 30 ml/h; void volume (V_0), 22 ml; column, 1.5 cm × 30 cm.

Fig. 2. Chromatography of 1 µmole each of asparagine[1]-angiotensin II, bacitracin, 5 mg $Cu(NO_3)_2$ (forming a complex with pyridine), and 5 mg NaCl on polyacrylamide gel (Bio-Gel, P-2). Eluting solvent, 0.1 *M* pyridinium acetate, pH 5.5; 2-ml fractions; flow rate, 30 ml/h; void volume (V_0), 22 ml; column, 1.5 cm × 30 cm.

Sephadex G-15

With 0.1 *M* pyridinium acetate at pH 5.5 asparagine[1]-angiotensin could be separated from bacitracin and from sodium ion but emerged in the same position as histidine and was therefore grossly retarded (Fig. 3). In 0.01 *N* HCl angiotensin amide and histidine also overlapped but there was in addition marked broadening of the descending limb of the angiotensin amide peak. Furthermore, there was overlap of histidine (plus angiotensin amide) with the sodium ion peak. In 0.01 *N* NH_4OH (pH 9.3–9.6) angiotensin amide emerged much later than at pH 5.5 or in HCl and appeared in the same fractions as sodium ion.

When aspartic acid[1]-angiotensin (asp[1]-ile[5] or the corresponding ^{14}C-labeled compound) was filtered through G-15 in 0.01 N NH_4OH, a condition in which asparagine[1]-angiotensin was retarded, both aspartic acid compounds appeared unexpectedly early and, indeed, just behind the void volume. When asp[1]-val[5]-angiotensin and asn[1]-val[5]-angiotensin were mixed and passed through G-15 under these conditions the two peptides, differing only by the amino acid in the first position, readily separated (Fig. 4). A mixture of stable asp[1]-ile[5]-angiotensin and asn[1]-val[5]-angiotensin was also separated. In two successive experiments ^{14}C-labeled aspartic acid[1]-angiotensin and stable asparagine[1]-angiotensin were compared in the presence and absence of 2 M NaCl added to the NH_4OH (Fig. 5). In the presence of the salt, both compounds were markedly retarded, but separation was still evident. When the filtration was performed in 0.01 N NH_4OH containing 1 M pyridine the separation of aspartic acid[1]-angiotensin and asparagine[1]-angiotensin appeared to be abolished, as the asparagine[1]-angiotensin appeared earlier than in the absence of the pyridine. Actually, as shown by simultaneous filtration of ^{14}C-labeled aspartic acid[1]-angiotensin and stable asparagine[1]-angiotensin, the latter was still slightly retarded relative to the aspartic acid compound. Prolonged washing of the column with pyridine did not prevent subsequent separation of the two angiotensins provided that the pyridine was washed out prior to their refiltration in NH_4OH.

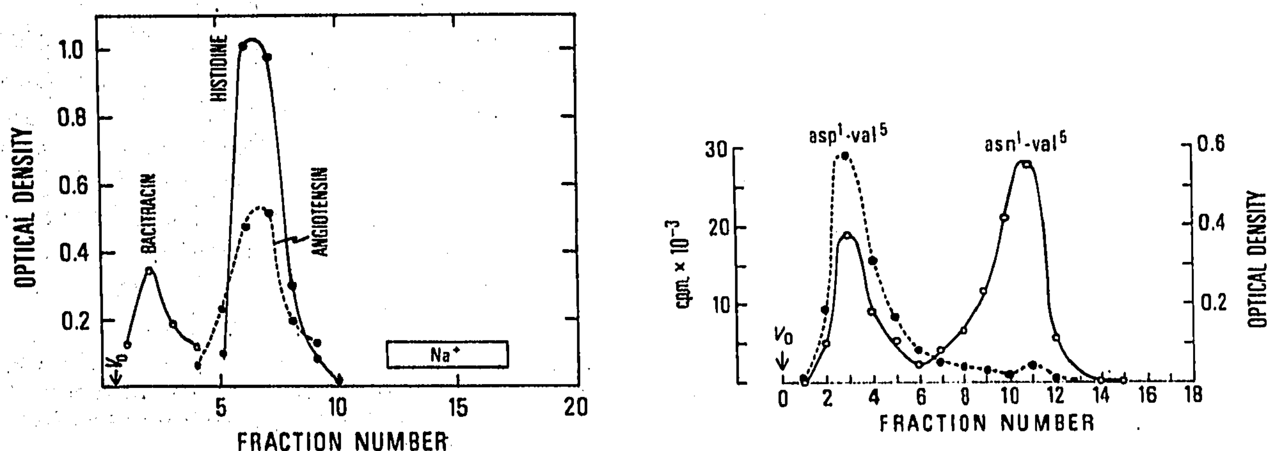
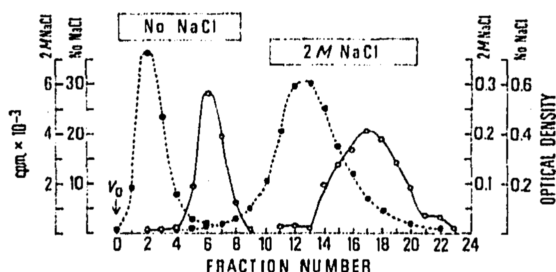


Fig. 3. Chromatography of bacitracin (1 μmole), histidine (0.6 μmole), asparagine[1]-angiotensin II (1 μmole), and NaCl (5 mg) on dextran gel (Sephadex G-15). Eluting solvent, 0.1 M pyridinium acetate, pH 5.5; 3-ml fractions; flow rate, 20 ml/h; void volume (V_0), 28 ml; column, 1.5 cm × 30 cm.

Fig. 4. Chromatography of asp[1]- and asn[1]-angiotensin II on Sephadex G-15 in 0.01 M NH_4OH. ●—●, ^{14}C-labeled angiotensin, 0.1 μCi; ○—○, asp[1]-val[5]-angiotensin II, 0.6 mg, and asn[1]-val[5]-angiotensin II, 1.2 mg. 1.5-ml fractions; flow rate, 4 ml/h; void volume (V_0), 18 ml; column, 0.9 cm × 68 cm.

Chromatography of [^{14}C]asparagine and [^{14}C]aspartic acid showed that both compounds emerged near the void volume in 0.01 N NH_4OH on G-15. Addition of 2 M NaCl showed the emergence, but in neither case was separation of the two compounds observed.



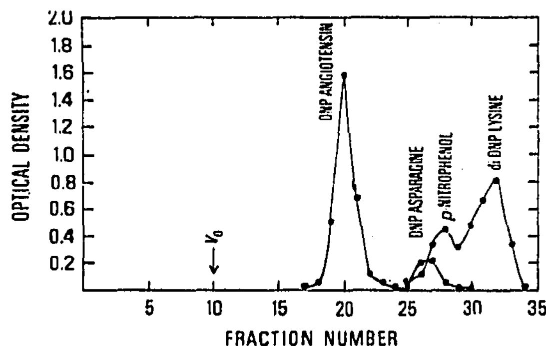


Fig. 5. Chromatography of ^{14}C-labeled asp^1-angiotensin and asn^1-angiotensin in the presence and absence of 2 M NaCl on Sephadex G-15 in 0.01 M NH_4OH. ●—●, ^{14}C-labeled asp^1-ile^5-angiotensin; ○—○, asn^1-val^5-angiotensin, 1.1 mg. 2.0-ml fractions; flow rate, 6 ml/h; void volume (V_0), 18 ml; column, 0.9 cm × 68 cm.

Fig. 6. Chromatography of DNP-angiotensin II, DNP-asparagine, and ε,N-di-DNP-lysine on alkylated dextran gel (Sephadex LH-20). Eluting solvent, absolute methanol, 0.05 M in pyridinium acetate; 3-ml fractions; flow rate, 30 ml/h; void volume (V_0), 30 ml; column, 1.5 cm × 30 cm.

Sephadex LH-20

Gel filtration of a mixture of the tri-DNP derivative of asparagine^1-angiotensin and DNP-asparagine in methanol showed good separation of the two compounds. A larger DNP-amino acid, ε,N-di-DNP-lysine, emerged later than DNP-asparagine (Fig. 6). An elution position identical to that for DNP-angiotensin was obtained when a microgram amount of ^{14}C-labeled DNP-angiotensin methyl ester was filtered in methanol, 0.05 M in pyridinium acetate.

Quantitation of recovery

When µmole (1 mg) amounts of angiotensin were quantitated by the diazonium color reaction, recovery was essentially quantitative. Furthermore, when 1 µg of asparagine^1-angiotensin was filtered through polyacrylamide with pyridinium acetate (*cf.* Fig. 2) and the quantitation made by double isotope derivative assay, 83 % was recovered. However, under the same conditions when 50 ng were passed through the P-2 column, nothing whatever could be recovered.

With 0.75 µg amounts of ^{14}C-labeled DNP-asparagine^1-angiotensin methyl ester passed through Sephadex LH-20 in methanol, quantitative recoveries were observed.

DISCUSSION

Although adsorption effects are well recognized for several types of compounds (amino acids, nucleotides, nucleosides, peptides) during gel chromatography on polyacrylamide and dextran gels[3–8], insufficient information is presently available to allow one to predict the degree of retardation one will observe for a specific small molecular weight peptide, although it has been previously established that acidic, basic, and, especially, aromatic amino acid components are important determinants of adsorption behavior.

Our interest in this problem arose from an examination of the possibility of incorporating molecular sieve gel chromatography into a method for the determination of angiotensin in biological materials, because of the desirability of separating the peptide from larger peptides and smaller molecules, especially the amino acids.

Initially we studied the synthetic octapeptide with sequence Asn-Arg-Val-Tyr-Val-His-Pro-Phe which differs from natural bovine angiotensin only by the presence of asparagine rather than aspartic acid at the N-terminus. Since the peptide contains two basic amino acids (arginine and histidine) in addition to the N-terminal amino group and two aromatic amino acids, the occurrence of adsorption was not especially remarkable or entirely unexpected. On the other hand, the degree of adsorption, its alteration with pH, and some significant differences between the adsorption seen on polyacrylamide as opposed to that on dextran gel does deserve some comment.

With polyacrylamide retardation of a basic (arginine) and two aromatic amino acids (phenylalanine and tryptophan) has been described[5]. In the case of arginine the presence of pyridine served to retard migration even further, although distinction between pH and solute effect was not made. Despite the prediction that adsorption phenomena might be seen with peptides on polyacrylamide[5], no such observations appear to have been reported heretofore. In the present studies with angiotensin marked retardation was observed in dilute acid (0.05 N HCl). The effect was clearly not due to ion exchange, since retardation was not decreased by addition of 1 M sodium chloride, and unlike the effect of salt on adsorption of certain amino acids or peptides containing aromatic amino acids on dextran gels, was not increased either[7-9]. In fact, the position of the angiotensin peak was the same in 1 M NaCl (without acid) as in pyridinium acetate, and in both of the latter circumstances little or no unusual retardation was seen. It seems, therefore, that low pH is the factor responsible for the strong adsorption of angiotensin on polyacrylamide. The possible mechanism of alteration of adsorption effects by variation of pH has been discussed by JANSON[9].

Filtration of asparagine[1]-angiotensin on dextran gel (Sephadex G-15) yielded results indicative of even greater retardation due to adsorption than was the case with polyacrylamide. The retardation was seen not only in acid but in pyridinium acetate (pH 5.5) as well, while at alkaline pH the salt peak and angiotensin emerged in the same fractions. However, at the beginning of our work only asparagine[1]-angiotensin was available to us. When the stable and ^{14}C-labeled aspartic acid-angiotensin octapeptides became available we decided to compare them with the asparagine material on G-15. We were surprised to observe a striking difference between the aspartic acid compounds and the asparagine derivative, separation being readily achieved at alkaline pH (Fig. 4). Clearly, the adsorption exhibited by the asparagine peptide was not shown by the aspartic acid analog.

The separation of asparagine- and aspartic acid-angiotensin is best explained by the considerations presented by EAKER AND PORATH for the behavior of amino acids on Sephadex G-10[7]. These workers showed clearly that net charge is an important determinant of adsorption behavior. It would appear that the greater negative charge of the aspartic acid[1]-angiotensin is sufficient to overcome the aromatic adsorption exhibited by the remainder of the molecule, *i.e.* by the asparagine congener. When filtration was performed in NH_4OH containing pyridine (1 M) the asparagine[1]-angiotensin and the aspartic acid peptides were no longer separable, although the former did emerge slightly behind the aspartic acid[1] compound. Pyridine, at this concentration, appeared to decrease greatly adsorption of the asparagine compound, as one would expect if aromatic adsorption were the mechanism of retardation of this peptide. Since the pyridine effect was reversible, removal from the gel of an unknown

anion with cation-exchange properties was not related to the observed effects in these experiments, which thus differ from those reported by EAKER AND PORATH for their supply of G-10[7]. Furthermore, the presence of a high concentration of salt (2 *M* NaCl) enhanced the adsorption of both compounds, but they were still separable (Fig. 5). This result makes it unlikely that the separation obtained at low ionic strength is due only to direct electrostatic exclusion and adsorption determined by fixed carboxyl groups on G-15.

The exclusion of negatively charged solutes on Sephadex G-10 at alkaline pH was shown by EAKER AND PORATH to involve not only molecules carrying more than one negative charge, such as glutamic and aspartic acids, but even molecules such as glycine, a finding not stressed by those authors. We are not aware of comparable data for larger molecules on G-15. Since aspartic acid[1]-angiotensin has a molecular weight of close to 1000 it is not remarkable that it should emerge near the void volume, considering the exclusion limit of G-15 of about 1500, but we were surprised to find that even the amino acids aspartic acid and asparagine (mol. wt. 133 and 132) were almost completely excluded in 0.01 *M* NH_4OH on G-15. Furthermore, aspartic acid and asparagine were not separable on G-15 under these conditions. It is clear from this observation that negative charge imparts a strong exclusion effect with G-15 even for molecules as small as amino acids. This consideration makes understandable the contribution of the negatively charged β-carboxyl group of the N-terminal aspartic acid of angiotensin toward overcoming the aromatic adsorption contributed by the remainder of the molecule.

The possibility of separating DNP-angiotensin from DNP-amino acids was explored because of our use of the DNP derivative in a double isotope assay for peptides[1]. DNP-angiotensin is almost completely insoluble in water. Gel chromatography was possible, however, using methanol as solvent and the alkylated Sephadex LH-20. Good separation was readily achieved between DNP-angiotensin amide, a mono-DNP-amino acid (asparagine), and a di-DNP-amino acid (lysine) (Fig. 4). The role of adsorption was not explored under these circumstances although clearly it was operative to at least some extent with the smaller compounds as well, since the di-DNP-lysine emerged later than the smaller DNP-aspartic acid and contaminating 2,4-dinitrophenol. In our experiments with polyacrylamide striking retardation due to adsorption was also observed with DNP-aspartic acid (Fig. 1) as had been seen earlier by others with DNP-ethanolamine[6].

No use was made in our work of the phenol solvent system in which adsorption can be abolished on Sephadex[8,10]. This approach is useful for the determination of peptide molecular size, but the solvent is relatively non-volatile and would present practical problems in the concentration of column effluents and recovery of the peptides.

REFERENCES

1 R. I. GREGERMAN AND M. A. KOWATCH, *Biochem. Med.*, 3 (1969) 13.
2 D. ABRAHAM, J. J. PISANO AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 99 (1962) 210.
2a J. BJERRUM AND E. J. NIELSEN, *Acta Chem. Scand.*, 2 (1948) 297.
3 B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
4 A. N. SCHWARTZ, A. W. G. YEE AND B. A. ZABIN, *J. Chromatog.*, 20 (1965) 154.
5 A. N. SCHWARTZ AND B. A. ZABIN, *Anal. Biochem.*, 14 (1966) 321.
6 J. S. FAWCETT AND C. J. O. R. MORRIS, *Separation Sci.*, 1 (1966) 9.
7 D. E. EAKER AND J. PORATH, *Separation Sci.*, 2 (1967) 507.
8 R. K. BRETTHAUER AND A. M. GOLICHOWSKI, *Biochim. Biophys. Acta*, 155 (1968) 549.
9 J. JANSON, *J. Chromatog.*, 28 (1967) 12.
10 P. R. CARNEGIE, *Nature*, 206 (1965) 1128.