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GEL FILTRATION OF PROTEINS AND PEPTIDES IN THE PRESENCE OF 6 *M* GUANIDINE HYDROCHLORIDE

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SUMMARY

It has been found that by using gel permeation chromatography both protein and peptide molecular weights can be determined on a single column of agarose equilibrated with 6 *M* guanidine hydrochloride. This technique has been successfully applied to the solution of a problem which is of particular interest to us. The fact that it was possible not only to determine the sub-unit molecular weight for horse spleen apoferritin but also the molecular weights for each of the peptides obtained from cyanogen bromide cleavage of the protein attests to the versatility of the method.

INTRODUCTION

The iron of ferritin, the principal iron storage protein of mammals, can be removed by reduction to give a protein shell, apoferritin[1–3]. The molecular weight of this apoprotein has been shown by a number of techniques to be in the range 430,000–465,000 (refs. 4–6). It has further been shown that the protein could be dissociated into subunits of molecular weight about 25,000 (ref. 7).

In the course of structural studies on this protein, cleavage with cyanogen bromide was carried out. Since the amino acid composition, based on a subunit molecular weight of 25,000, would indicate the presence of four methionine residues (being the nearest integral value) per subunit[8], it was anticipated that five peptides would be found after specific cleavage at the methionyl residues. However, when the cleavage was performed only four peptides were found[9]. Further, it was shown that quantitative N-terminal determinations on the total cyanogen bromide cleavage mixture, by the method of STARK AND SMYTH[10], revealed only three new end groups, and these were found in equimolar amounts[11]. This is what would be expected from four peptides, since it is known that the N-terminal residue of apoferritin is acetylated[12].

Re-determination of the subunit molecular weight in this laboratory by a number of techniques gave a value of 18,500 (refs. 13 and 14). The amino acid composition based on this value indicates that there are three methionine residues

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per subunit, which is consistent with four peptides from cyanogen bromide cleavage, and this seems to resolve the previously conflicting data.

In the course of determining the subunit molecular weight by chromatography on an agarose column in buffers containing 6 *M* guanidine hydrochloride by Dawson's[15] method, as modified by FISH *et al.*[16], we found that it was also possible to determine peptide molecular weights with the same column. Molecular weights in the range 1,400–8,000 could be determined with an accuracy of better than 10%. We report here the application of this method to the estimation of the molecular weights of the peptides of horse spleen apoferritin obtained by cyanogen bromide cleavage.

Apart from these analytical applications we have also used the technique on a preparative scale and as a test for homogeneity of samples prior to sequence studies which, together with the speed and reproducibility, testify to the potential of the technique.

A preliminary report of this work has already been presented[17].

MATERIALS AND METHODS

Glucagon, apoferritin (horse spleen), catalase (beef liver) and gramicidin were obtained from Mann Research Laboratories, New York, N. Y.; alcohol dehydrogenase (liver), chymotrypsinogen A, ovalbumin, trypsin and lima bean trypsin inhibitor from Worthington Biochemical Corporation, Freehold, N. J.; creatine phosphokinase (rabbit muscle), cytochrome C (horse heart), haemoglobin and insulin from Sigma Chemical Co., St. Louis, Mo.; ferritin (horse spleen) and transferrin (horse, pooled plasma) from Koch Ligh Laboratories Ltd., Colnbrook, Great Britain; albumin (bovine plasma) from Armour Pharmaceutical Co. Ltd., Eastbourne, Great Britain; apoferritin (horse spleen) and globulin (porcine) from Rentex Inc., Kankakee, Ill., myoglobin (horse heart) from British Drug Houses Ltd., Poole, Great Britain; and bacitracin and insulin (bovine pancreas) from Schwarz/Mann, Orangeburg, N. Y. Sepharose 6B (Lot No. 5073) with a nominal agarose content of 6% was obtained from Pharmacia, Uppsala, Sweden while Bio-Gel A-5m (100–200 mesh, control No. 49612) was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Preparation of peptides using cyanogen bromide

Cyanogen bromide cleavage of apoferritin and the other proteins was carried out as reported elsewhere[9,18] and the resulting peptide mixtures re-lyophilised from water several times in order to remove completely any excess reagent or volatile by-products.

Gel filtration on agarose

Agarose is a linear polysaccharide consisting of alternate residues of D-galactose and 3,6-anhydro-L-galactose. Although there are no covalent cross-linkages stabilising the Sepharose matrix, it does not seem to be affected by high concentrations of salt provided great care is taken during gel equilibration to avoid hydrolysis of the glycosidic linkages. We have already described the experimental procedure for column preparation, gel equilibration, sample preparation and application and also for guanidine hydrochloride purification[14]. It should be pointed out that all samples were reduced and carboxymethylated prior to chromatography.

RESULTS

Chromatographic resolution

Effect of flow rate. Fig. 1 shows the effect of the flow rate on the resolution obtained by chromatography of identical samples of marker proteins and peptides. It can be seen from the elution profiles that the resolution increases with decreasing flow rates and that adequate resolving power without significant zone broadening can be attained with a flow rate of 2 ml/h.

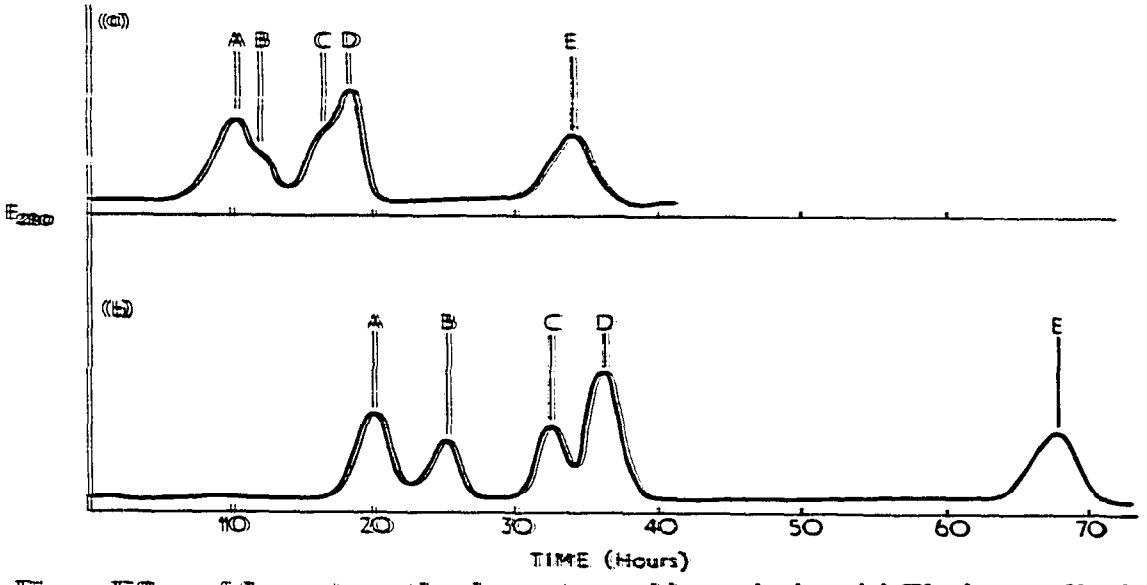

Fig. 1. Effect of flow rate on the chromatographic resolution. (a) Elution profile obtained following fractionation at 4 ml/h. (b) Elution profile obtained following chromatography of an identical sample at 2 ml/h. A = Bovine serum albumin; B = ovalbumin; C = horse heart myoglobin; D = cytochrome C; E = tryptophan.

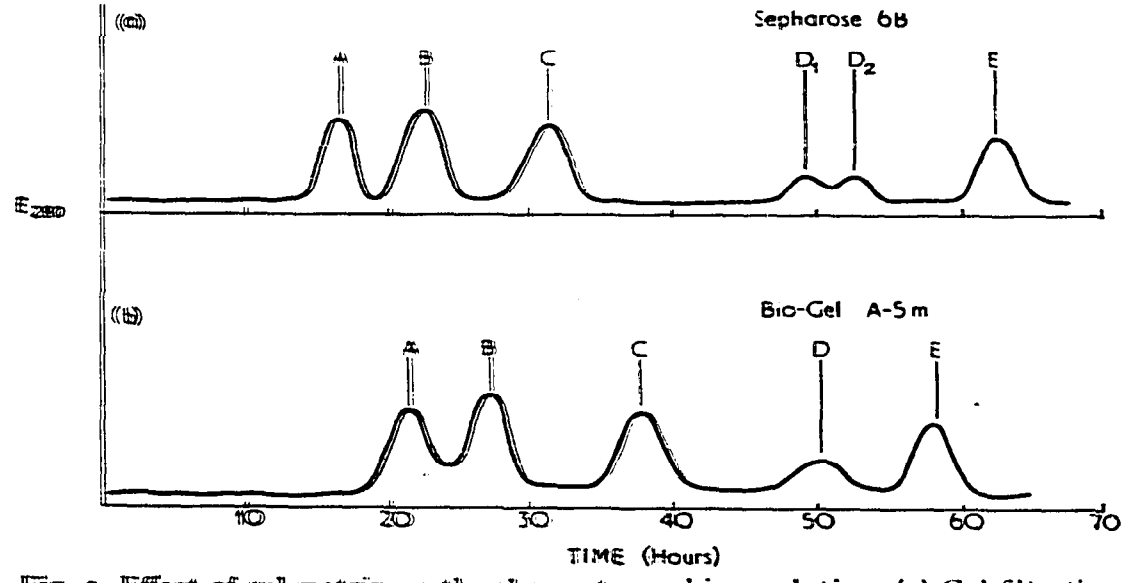

Fig. 2. Effect of gel matrix on the chromatographic resolution. (a) Gel filtration on Sepharose 6B. (b) Gel filtration of an identical sample on Bio-Gel A-5m. A = Transferrin; B = alcohol dehydrogenase; C = haemoglobin; D = insulin; D_1 = insulin, B chain; D_2 = insulin, A chain; E = tryptophan.

Effect of gel matrix. Fig. 2 shows two typical elution profiles for the same sample on two different molecular sieve matrices following chromatography under similar conditions. From this it can be seen that for the molecular weight range 11,000–80,000 there is little difference in the elution pattern. For molecular weights less than 9,000, however, it is apparent that the resolving power of the Sepharose is much greater than that of the Bio-Gel equivalent. Whereas on Bio-Gel A-5m the two chains of insulin elute as one unresolved peak, on Sepharose 6B the two chains can be easily separated. FISH *et al.*[16] previously noted that insulin "eluted as a broad peak" on a column of Bio-Gel A-5m.

Treatment of data

In all these studies we describe the chromatographic behaviour of the proteins/peptides by the parameter K_{av} [19], where

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

V_0, the exclusion volume, is obtained by co-chromatography of the sample with Dextran Blue 2000, which is completely excluded from the gel matrix, while V_t, the total volume accessible to the solvent, is obtained by co-chromatography of the sample with tryptophan. The elution volume, V_e, is the position at which the protein/peptide elutes following gel filtration. Once this parameter has been determined

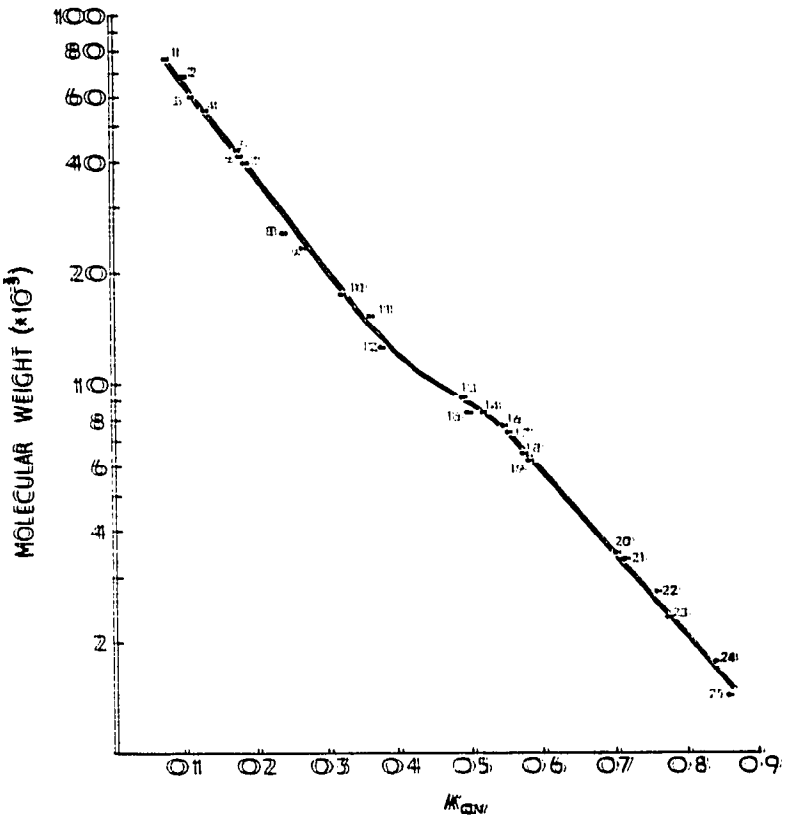

Fig. 3. Molecular weight determination by gel filtration on Sepharose 6B in the presence of 6 *M* guanidine hydrochloride. Distribution coefficient (K_{av}) is shown as a function of molecular weight and the linear relationships were computed by the method of least squares analysis. The numbers refer to the proteins and peptides listed in Table I.

it could be related to the molecular weight by either the empirical relationship of ANDREWS[20]

$$K_{av} = a - b \lg M$$

or the theoretical treatment of PORATH[21]

$$K_{av}^{1/3} = k_1 - k_2 M^{1/2}$$

where a, b, k_1 and k_2 are constants and M denotes molecular weight.

Regression analysis of the experimental data was obtained from computations using a PDP-8/L computer (Digital Equipment Corporation, Reading, Great Britain).

Fig. 3 shows the plot of the distribution coefficient, K_{av}, *versus* the logarithm of molecular weight for the molecular weight markers used in this study (see also Table I). It can be seen from this empirical relationship that there are two regions of apparent linearity, one in the molecular weight range 11,000–80,000 and the other in the range 1,400–8,000. These linear relationships can be expressed in the forms

$$\log M = 5.091 - 2.652 K_{av}$$

and

$$\log M = 5.176 - 2.357 K_{av}$$

respectively.

TABLE I

PROTEINS AND PEPTIDES USED AS MOLECULAR WEIGHT MARKERS IN THIS STUDY

No.	*Protein/peptide*	*Molecular weight*	K_{av}	$(Mol.\ wt.)^{1/2}$	$K_{av}^{1/3}$
1	Transferrin	76,600	0.0758	276.77	0.4236
2	Serum albumin	68,000	0.1024	260.77	0.4682
3	Catalase	60,000	0.1144	244.95	0.4857
4	γ-Globulin, H chain	55,000	0.1343	234.52	0.5124
5	Ovalbumin	43,000	0.1769	207.36	0.5616
6	Alcohol dehydrogenase (liver)	41,000	0.1795	202.48	0.5644
7	Creatine phosphokinase	40,000	0.1875	200.00	0.5727
8	Chymotrypsinogen	25,700	0.2380	160.31	0.6200
9	γ-Globulin, L chain	23,500	0.2673	153.30	0.6445
10	Myoglobin	17,200	0.3218	131.15	0.6855
11	Haemoglobin	15,500	0.3630	124.50	0.7136
12	Cytochrome C	12,300	0.3763	110.91	0.7222
13	Trypsin — CNBr I	9,209	0.4893	95.96	0.7882
14	Lima bean trypsin inhibitor	8,400	0.5141	91.65	0.8012
15	Myoglobin — CNBr I	8,181	0.4960	90.45	0.7918
16	Cytochrome C — CNBr I	7,733	0.5492	87.94	0.8191
17	Trypsin — CNBr II	7,536	0.5518	86.81	0.8204
18	Trypsin — CNBr III	6,533	0.5731	80.83	0.8308
19	Myoglobin — CNBr II	6,235	0.5784	78.96	0.8333
20	Glucagon	3,480	0.7021	58.99	0.8889
21	Insulin, B chain	3,400	0.7127	58.31	0.8933
22	Cytochrome C — CNBr II	2,780	0.7579	52.73	0.9118
23	Insulin, A chain	2,340	0.7739	48.37	0.9182
24	Cytochrome C — CNBr III	1,780	0.8470	42.19	0.9462
25	Bacitracin	1,411	0.8563	37.56	0.9497

Fig. 4 shows the data plotted according to PORATH's theoretical treatment for random coils[21] (see also Table I). From this it can be seen that there are essentially two linear relationships and these are defined as shown in the figure.

One possible explanation for an empirical relationship of this nature, in view of earlier studies by DETERMANN and others[19,20,22], would be to suppose the presence of two populations of gel matrix differing only in the nature of their porosities. DETERMANN AND MICHEL[22] and LAURENT AND KILLANDER[19] have observed that, for a given molecular weight, the K_{av} increases with a decreasing degree of cross-linking over the range of the Sephadex series. Extrapolating the results of these studies to our experimental data for agarose it would seem possible that in the microstructure of the starting material there was a pore size distribution such that the material with the greater extent of cross-linking was involved in the peptide chromatography while that with a somewhat lesser degree of cross-linking was responsible for fractionation of the polypeptide chains. Using this assumption it can be seen from the general form of the equation

$$\log M = C_1 - C_2 K_{av}$$

that for the region of protein chromatography (11,000–80,000 Daltons) the absolute value for C_2 is greater than the corresponding value for the linear region involved in peptide separation (1,400–8,000 Daltons) and from the general equation in the other form

$$K_{av}^{1/3} = C_1 - C_2 M^{1/2}$$

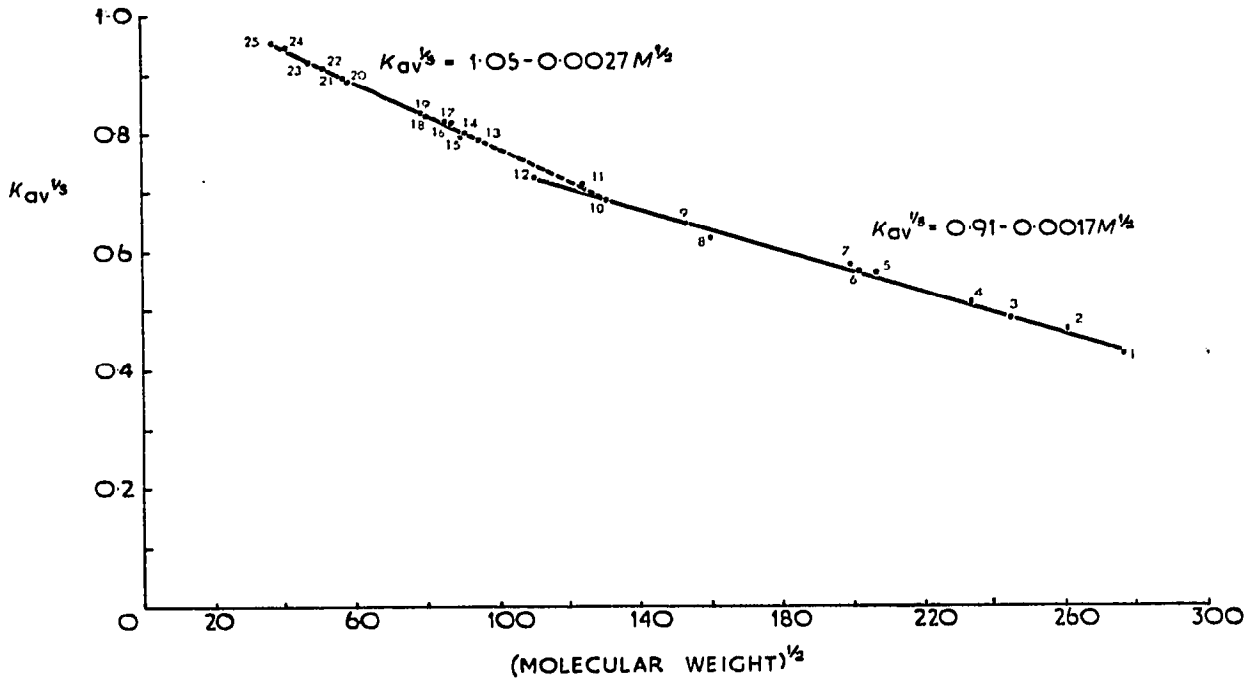

Fig. 4. Molecular weight determination by gel filtration on Sepharose 6B in the presence of 6 *M* guanidine hydrochloride. The figure shows the treatment of the experimentally determined distribution coefficients according to the method of PORATH. $K_{av}^{1/3}$ is plotted as a function of (molecular weight)$^{1/2}$ and the linear relationships were computed by regression analysis as before. The numbers refer to the proteins and peptides listed in Table I.

the converse is true. Both these findings are consistent with the experimental data obtained by other workers[20,22].

The reason for the differences in resolution in the low-molecular-weight region (1,400–8,000 Daltons) between Sepharose 6B and the Bio-Gel equivalent may merely reflect a difference in the preparation of the agarose spheres.

Since it is known that the degree of cross-linking for agarose is attributable to hydrogen bonding and that a high concentration of guanidine hydrochloride can cause destruction of hydrogen bonds, it is surprising that any such separation should take place at all.

One problem of such gel filtration columns is that the flow rate decreases slowly with time and in some extreme cases the column flow can even stop completely, although if care is taken at the equilibration stage the latter is seldom, if ever, observed. This is almost certainly a reflection of high salt degradation of the gel matrix resulting in the generation of fines. Despite the slowly changing flow characteristics, however, there was no discernible effect on the K_{av} values.

Recently AFANAS'EV derived an equation for the dependence of the logarithm of the distribution coefficient of the protein upon its molecular weight to the power $^2/_3$ from consideration of the surface energy at the protein–solvent interface and the distribution of substances in a two-phase system[23]. The relationship which he found from such theoretical considerations is given by the following equation:

$$\ln (1/K_d) = \frac{4.84((\sigma_1 - \sigma_2))N^{1/3}V^{2/3}}{RT} \cdot M^{2/3}$$

or, more generally, for a given system as:

$$\ln (1/K_d) = A \cdot M^{2/3}$$

where A is constant.

It would be expected therefore that when a graph is drawn with co-ordinates $M^{2/3}$ and log $(1/K_d)$ a straight line passing through the origin would be obtained. Fig. 5 shows the results of plotting our experimental data in this manner. Once again two linear relationships were obtained but neither of these straight lines passed through the origin. We found it possible to explain the empirical relationships obtained from such treatment of our data by once again invoking the concept of two gel porosities.

For the gel population of lower porosity. If we assume that the fraction of the total gel matrix that is involved in protein chromatography is C, then we have:

$$K_{av}(\text{empirical}) = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

and

$$K_{av}(\text{theoretical}) = \frac{[V_e - ((1 - C)V_0)] - CV_0}{CV_t - CV_0} \quad (2)$$

where K_{av} (empirical) is the K_{av} value determined experimentally and K_{av} (theoretical) is the true value for the K_{av} in the gel matrix that is involved in polypeptide gel filtration. Thus from (2)

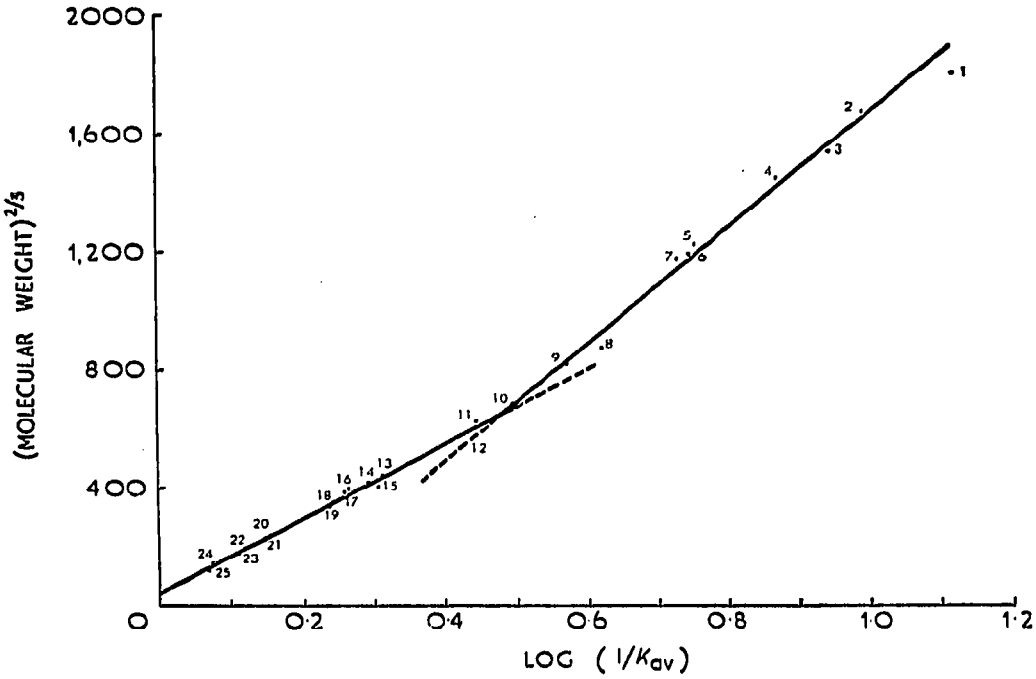

Fig. 5. Molecular weight determinations by gel filtration on Sepharose 6B in the presence of 6 *M* guanidine hydrochloride. The figure shows the treatment of the experimental data according to the method of AFANAS'EV. (Molecular weight)$^{2/3}$ is plotted as a function of log ($1/K_{av}$) and the linear relationships were computed by regression analysis as before. The numbers refer to the proteins and peptides listed in Table I.

$$K_{av}(\text{theoretical}) = 1/C \cdot \frac{V_e - V_0}{V_t - V_0}$$
$$= 1/C \cdot K_{av}(\text{empirical})$$

hence

$$\log [1/K_{av}\,(\text{theoretical})] = \log (1/K_{av}\,(\text{empirical})] - \log (1/C)$$

Now from AFANAS'EV's equation

$$A \cdot M^{2/3} = \log [1/K_{av}\,(\text{theoretical})]$$

hence

$$M^{2/3} = 1/A \cdot \log [1/K_{av}\,(\text{empirical})] - 1/A \cdot \log (1/C)$$

From regression analysis of our data we obtained

$$M^{2/3} = 1894.16 \log (1/K_{av}) - 244.737$$

hence

$$\log (1/C) = \frac{244.737}{1894.16} = 0.1292$$

and

$$C = 0.7424$$

Thus from such computations it would seem that about 75 % of the gel matrix is responsible for the fractionation of the polypeptide chains.

For the gel population of greater porosity. If we assume that the use of tryptophan is not a true measure of the total volume accessible to the solvent (V_t) and that the theoretical value for this parameter is V, then we have

$$K_{av}\,(\text{empirical}) = \frac{V_e - V_0}{V_t - V_0} \tag{3}$$

$$K_{av}\,(\text{theoretical}) = \frac{V_e - V_0}{V - V_0} \tag{4}$$

$$K_{av}\,(\text{tryptophan}) = \frac{V_t - V_0}{V - V_0} \tag{5}$$

From eqn. (5) we have

$$V = \frac{V_t - V_0 + V_0\,K_{av}\,(\text{tryptophan})}{K_{av}\,(\text{tryptophan})}$$

Substituting this value in eqn. 4

$$K_{av}\,(\text{theoretical}) = \frac{V_e - V_0}{\dfrac{V_t - V_0 + V_0\,K_{av}\,(\text{tryptophan})}{K_{av}\,(\text{tryptophan})} - V_0}$$

$$= \frac{V_e - V_0}{V_t - V_0} \cdot K_{av}\,(\text{tryptophan})$$

hence

$$K_{av}\,(\text{theoretical}) = K_{av}\,(\text{empirical}) \cdot K_{av}\,(\text{tryptophan})$$

Substituting this relationship once again into AFANAS'EV's equation we have,

$$M^{2/3} = 1/A \cdot \log\,[1/K_{av}\,(\text{empirical})] + 1/A\,\log\,[1/K_{av}\,(\text{tryptophan})]$$

From regression analysis of our data we have,

$$M^{2/3} = 1265.8\,\log\,(1/K_{av}) + 43.603$$

Theoretically, the intercept on the y-axis should be the molecular weight of tryptophan to the power 2/3. Thus we would expect this value to be $(204.2)^{2/3}$, which is equal to 34.68. It can be seen that this value is close to the experimentally determined value of 43.603. This would appear to justify our use of tryptophan as a measure of K_{av} and indicate that there is very little, if any, adsorption.

The elution profile obtained by chromatography of the cyanogen bromide cleavage mixture of horse spleen apoferritin is shown in Fig. 6a. Also shown in this figure are the molecular weights computed for the various molecular species. From these computed values we found it difficult to combine any permutation of the peptides to give us the magnitude of the molecular weight of the apoferritin subunit[13,14]. In order to rationalise this situation we were obliged to assume the presence

of a small, as yet undetected, peptide. This peptide, we assumed, either contained no tryptophan or tyrosine or was sufficiently small to be included under the tryptophan peak. As a result we initially pooled the tryptophan peak and attempted to remove

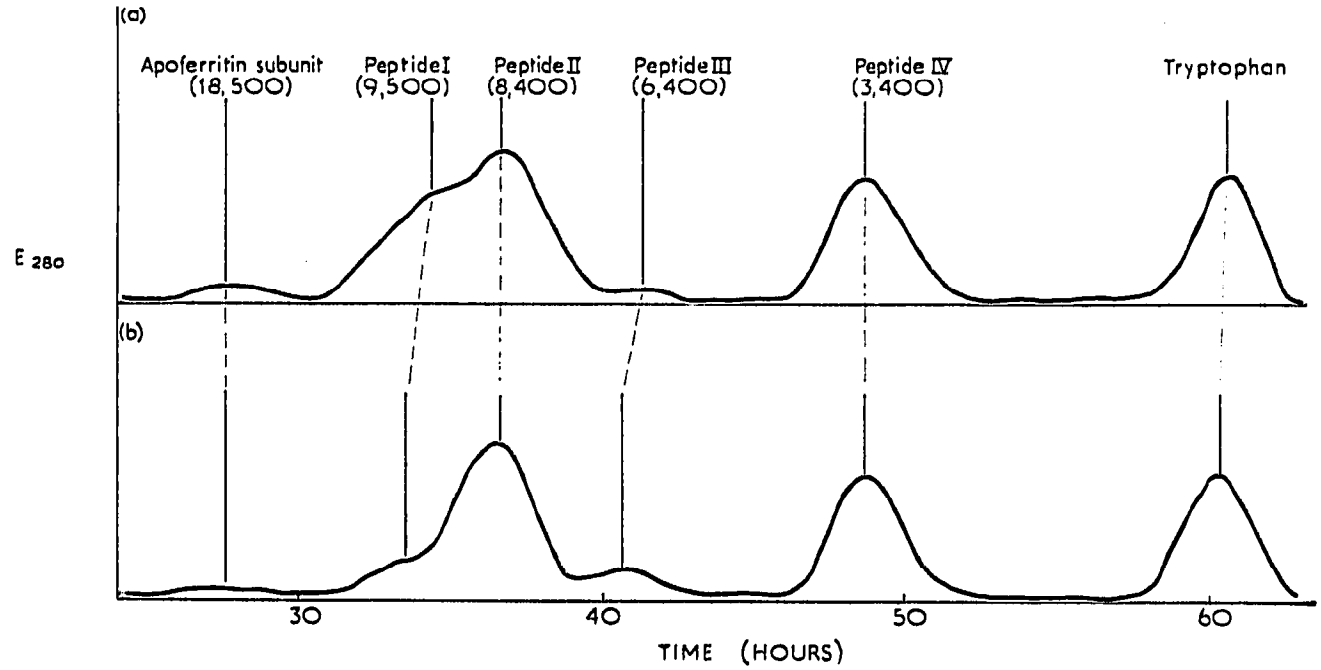

Fig. 6. (a) Elution profile obtained from fractionation of the cyanogen bromide cleavage products of horse spleen apoferritin on a column of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride, pH 5.0. (b) Elution profile obtained from fractionation of the cyanogen bromide cleavage products of horse spleen apoferritin by the modified method of chemical cleavage (see text).

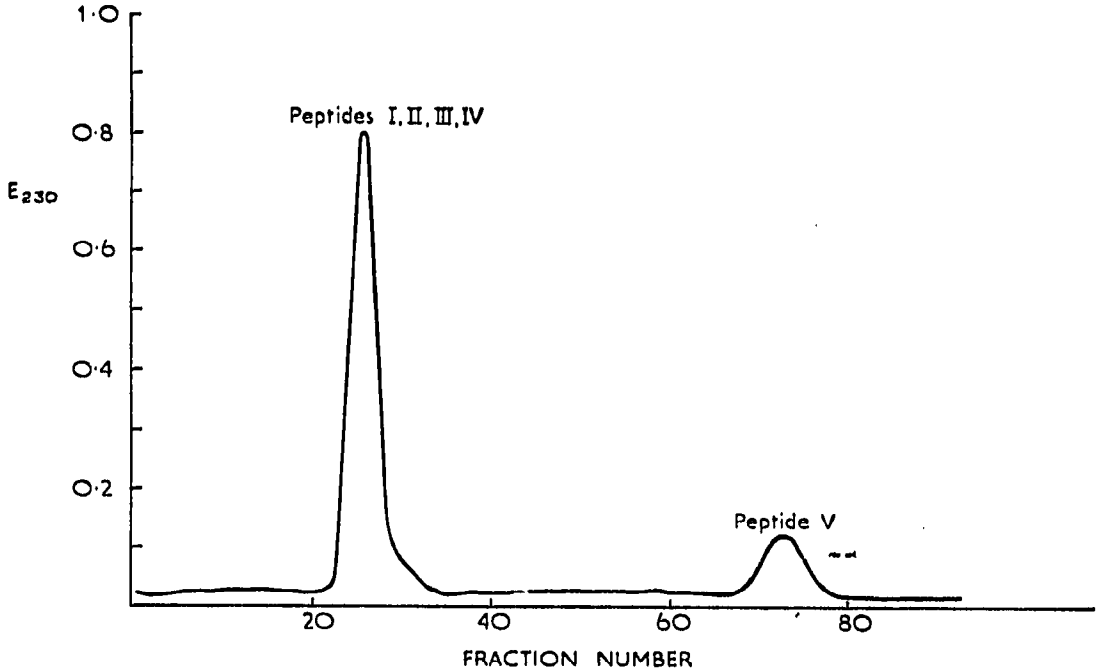

Fig. 7. Elution profile obtained from chromatography of the cyanogen bromide cleavage products of horse spleen apoferritin on a Sephadex G-15 column (2.5 cm × 45 cm) equilibrated with 1 *M* acetic acid. Peptides I, II, III and IV are eluted as one peak with the void volume while peptide V is retarded (see text for peptide nomenclature).

the guanidine hydrochloride by column chromatography on a Sephadex G-10 gel matrix. This proved to be a rather troublesome procedure and so it was decided to chromatograph the pure cyanogen bromide digest on a column of either Sephadex G-10 or G-15 equilibrated with 1 *M* acetic acid; the peptides were detected by their extinction at 230 or 235 nm. Fig. 7 shows the results of such a fractionation. The low-molecular-weight species so obtained was purified by further gel chromatography. Finally the pure peptide was hydrolysed with constant boiling 6 *N* HCl at 110° for 16 h and the resulting hydrolysate subjected to amino acid analysis using a Jeolco amino acid analyser (Japan Electron Optics Laboratory Co. Ltd., Chiyoda-ku, Tokyo). The composition of the peptide was found to be: Asx, 1.1; Glx, 0.98; Gly, 1.05; Leu, 1.0; Phe, 0.7; Ser; 0.7, HSer, present but not determined*. The peptide was purified to the extent that contaminating amino acids were present at less than 0.15 moles per mole of peptide. From these analyses the molecular weight of the peptide was calculated to be 900 Daltons.

We also found it possible to improve the resolution of the separate peptides by treating the protein, prior to the addition of the cyanogen bromide, for 20 h at 25° with a solution of 3 m*M* dithiothreitol/80 % (v/v) formic acid which had been saturated with nitrogen[24] (see Fig. 6b).

It can be seen now that the four peptides, II — 8,400 ± 400, III — 6,400 ± 300, IV — 3,400 ± 100 and V — 900, add up to give a molecular weight of 19,100 ± 800, which is in reasonable agreement with our previously determined value for the subunit molecular weight.

The two possible ways in which we can explain the origin of peptide I are shown in Fig. 8. This peptide (mol. wt. 9,500 ± 500), which contains an intact methionine, could on treatment with cyanogen bromide give rise to either peptide III (mol. wt. 6,400 ± 300) and peptide IV (mol. wt. 3,400 ± 100) or peptide II (mol. wt. 8,400 ± 400) and peptide V (mol. wt. 900). We are at present involved in establishing the alignment of these peptides in the subunit polypeptide chain and thereby should be able to distinguish between these two possibilities.

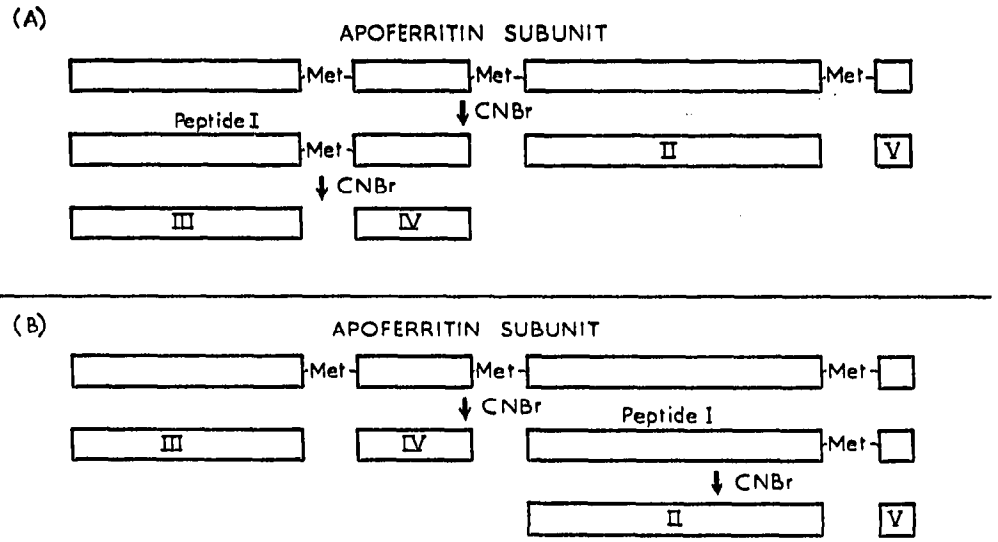

Fig. 8. Possible peptide alignments for the products of cyanogen bromide cleavage of horse spleen apoferritin.

* Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine; HSer, homoserine.

We found it possible to isolate each peptide preparatively by either one of two methods. The guanidine could be removed by column chromatography on a Sephadex G-10 gel matrix as described before for the small peptide V or, in the case of peptides I, II, III and IV, the salt could be removed by dialysis. The porosity of the dialysis tubing used in this way was decreased by acetylation with pyridine–acetic anhydride (3:1) for 8 h at 60°. The pyridine and acetic acid were then removed by extensive washing.

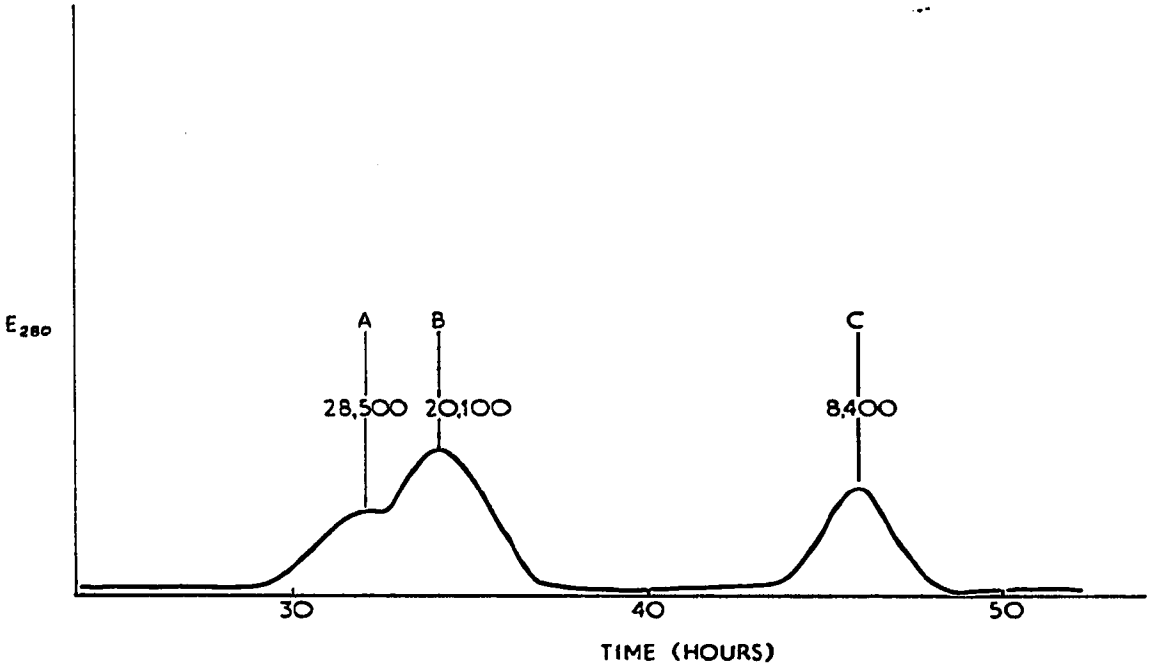

Fig. 9. Elution profile obtained from chromatography of a commercial preparation of lima bean trypsin inhibitor (Worthington Biochemical Corporation) on a column of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride, pH 5.0. The trypsin inhibitor activity is associated with peak C while peaks A and B merely represent commercial impurities.

A further example of the application of the method is given by the elution profile obtained for a commercial preparation of lima bean trypsin inhibitor (Worthington Biochemical Corp.) as shown in Fig. 9. It was found that all of the detectable trypsin inhibitor activity was associated with the lower-molecular-weight species (mol. wt. 8,400) and that the higher molecular-weight-species merely represented a commercial impurity[25,26]. By gel filtration in 6 *M* guanidine hydrochloride it was possible to resolve two impurities, I and II, the molecular weights of which were computed to be of the order of 28,500 and 20,100, respectively.

DISCUSSION

It has been observed by TANFORD[27] that all proteins so far studied in concentrated guanidine adopt a completely disordered state, the random coil conformation, and that the chain length of the resulting unfolded protein is a direct function of the molecular weight. In the context of gel filtration this must certainly be considered an asset since its use does not necessitate any consideration of the shape factor[20]. As it has been found that the ratio of the hydrodynamic volume of a protein in a random coil conformation to the hydrodynamic volume in its ordered structure is

about 10:1 (refs. 28 and 29) it would be expected that the operating range for the gel matrix would be decreased tenfold. This would result in the exclusion limit for Sepharose 6B being reduced to about 400,000. The actual exclusion limit we observed by extrapolation of the data in Fig. 4 is seen to be of the order of 120,000–150,000.

Recently a technique was developed for peptide chromatography and molecular weight determinations using an organic solvent system, phenol–acetic acid – water, as the denaturant[30]. The operating range was, however, limited to material of less than 35,000 Daltons. However, TANFORD[27] pointed out that such organic solvents do not give rise to a random coil conformation but rather to a state where there is a high helix content; this may thus explain why these workers found anomalous proteins.

Other workers have recently determined peptide molecular weights in the presence of the anionic detergent sodium dodecyl sulphate. These workers found that such determinations could be carried out with an accuracy of about 18 % although they also found anomalous peptides[31]. We have also attempted to characterise peptides by extending the range for SDS-polyacrylamide gel electrophoresis but, as we have already stated, the error in the estimation was such as to make a more intensive investigation unprofitable[14,32].

We have already mentioned that we have applied this technique preparatively for the isolation of peptides from protein digests. This has the advantage over fractionation carried out in normal, non-denaturant buffers in that it eliminates any specific or non-specific aggregation of material and thereby obviates recourse to extensive re-chromatography. Furthermore, as PIEZ and others (16, 33) have indicated, by continuous monitoring of the effluent, such techniques provide a sensitive and reliable visual estimate of polydispersity and heterogeneity.

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REFERENCES

1 S. GRANICK AND L. MICHAELIS, *J. Biol. Chem.*, 147 (1943) 91.
2 M. J. BIELIG AND E. BAYER, *Naturwissenschaften*, 42 (1955) 466.
3 A. MAZUR, A. CARLETON AND A. CARLESEN, *J. Biol. Chem.*, 236 (1961) 1109.
4 P. M. HARRISON, *J. Mol. Biol.*, 1 (1959) 69.
5 A. ROTHEN, *J. Biol. Chem.*, 152 (1944) 679.
6 G. W. RICHTER AND G. F. WALKER, *Biochemistry*, 6 (1967) 2871.
7 T. HOFMANN AND P. M. HARRISON, *J. Mol. Biol.*, 6 (1963) 256.
8 R. R. CRICHTON, *Biochim. Biophys. Acta*, 194 (1969) 34.
9 R. R. CRICHTON AND V. BARBIROLI, *FEBS Lett.*, 6 (1970) 134.
10 G. R. STARK AND D. G. SMYTH, *J. Biol. Chem.*, 238 (1963) 214.
11 R. R. CRICHTON, *Z. Physiol. Chem.*, 352 (1971) 9.
12 A. A. SURAN, *Arch. Biochem. Biophys.*, 113 (1966) 1.

13 R. R. CRICHTON AND C. F. A. BRYCE, *FEBS Lett.*, 6 (1970) 121.
14 C. F. A. BRYCE AND R. R. CRICHTON, *J. Biol. Chem.*, 246 (1971) 4198.
15 P. F. DAVISON, *Science*, 161 (1968) 906.
16 W. W. FISH, K. G. MANN AND C. TANFORD, *J. Biol. Chem.*, 244 (1969) 4989.
17 C. F. A. BRYCE AND R. R. CRICHTON, *Z. Physiol. Chem.*, 352 (1971) 9.
18 R. R. CRICHTON, *Biochem. J.*, 117 (1970) 34P.
19 T. C. LAURENT AND J. KILLANDER, *J. Chromatogr.*, 14 (1964) 317.
20 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
21 J. PORATH, *Pure Appl. Chem.*, 6 (1963) 233.
22 H. DETERMANN AND W. MICHEL, *J. Chromatogr.*, 25 (1966) 303.
23 P. V. AFANAS'EV, *Biokhimiya*, 35 (1970) 562.
24 G. R. DRAPEAU AND C. YANOFSKY, *J. Biol. Chem.*, 242 (1967) 5434.
25 G. JONES, S. MOORE AND W. H. STEIN, *Biochemistry*, 2 (1963) 66.
26 J. KRAHN AND F. C. STEVENS, *FEBS Lett.*, 13 (1971) 339.
27 C. TANFORD, *Adv. Protein Chem.*, 24 (1970) 1.
28 K. A. PIEZ AND A. L. CARILLO, *Biochemistry*, 3 (1964) 908.
29 M. S. LEWIS AND K. A. PIEZ, *Biochemistry*, 3 (1964) 1126.
30 A. PUSZTAI, AND W. B. WATT, *Biochim. Biophys. Acta*, 214 (1970) 463.
31 R. T. SWANK AND K. D. MUNKRES, *Anal. Biochem.*, 39 (1971) 462.
32 C. F. A. BRYCE AND R. R. CRICHTON, unpublished results.
33 K. A. PIEZ, *Anal. Biochem.*, 26 (1968) 305.