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APPENDIX

A Semi-Micro Method for the Determination of Amino Acids by Ion-Exchange Chromatography

By J. E. EASTOE

Department of Dental Science, Royal College of Surgeons of England, London, W.C. 2

(Received 31 October 1960)

The method of protein analysis, introduced by Moore & Stein (1951) and based on separation of amino acids on ion-exchange columns eluted with buffer solutions of accurately controlled pH, has become widely adopted. Collaborative tests have shown that, provided sufficient care is taken with all details of technique, the accuracy of $\pm 3\%$, originally claimed, can be attained for most amino acids (Bender, Palgrave & Doell, 1959). In some fields of investigation, e.g. purified enzymes and microbial toxins, enamel matrix (Eastoe, 1960*a*) and subcellular protein fractions, it is difficult or tedious to obtain even the 2–4 mg. of protein required for analysis by the standard method. A semi-micro method suitable for 0.3–0.6 mg. samples of protein was developed and has been described in a preliminary communication (Eastoe, 1960*b*). It is based on the original method of Moore & Stein (1951) with the minor changes suggested by Eastoe (1955). The modifications necessary for the semi-micro method, together with an indication of the accuracy and resolution obtainable, are described briefly below. It is not considered that this modification represents the ultimate in small-scale protein analysis, but is an exploratory step in that direction.

MATERIALS AND APPARATUS

Resin, buffers etc. The same batch of Dowex 50 resin (200–400 mesh; nominal 12% cross-linking) was used as previously (Eastoe, 1955). Citrate and phosphate buffers, thymol, BRIJ 35, thiodiglycol, ninhydrin and ethylene glycol monomethyl ether were prepared or purified as before. Industrial spirit (74 over proof) has been found to be a satisfactory substitute for absolute ethanol in the preparation of the diluent solution. The semi-micro method is considerably more economical in ninhydrin and buffer

solutions than the original method and it is therefore unnecessary to store large volumes of solutions.

Columns. The borosilicate glass tubes for the 15 and 100 cm. resin columns were 28 and 112 cm. in overall length respectively. They were selected for straightness and for an internal diameter of 4.0 ± 0.1 mm. (external diam. approx. 6.8 mm.). Each tube had a fused-in sintered-glass disk (porosity 1 or 2), 2.0 cm. from its lower end, which was cut off at 45° and its tip fused to a small blob of glass. Care was necessary to ensure that the disk was fused to the wall all round but not fused solid itself. The portion of tube below the sinter did not normally remain filled with solutions which contained detergent. The tubes were fitted by means of rubber bungs into side-armed glass jackets of overall length 18 and 102 cm. respectively. The columns were filled with resin as described by Moore & Stein (1951) with additional precautions to prevent trapping of air bubbles. The tubes were freed from grease with nitric acid–chromic acid and well rinsed with water and the walls left wet. The resin suspension was poured into the accurately vertical tubes very carefully to avoid air being trapped. When this happened, the bubbles were immediately shifted by prodding with a piece of 20-gauge copper wire.

A small column, for preliminary separation of basic amino acids, had an overall length of 8 cm. and an internal diameter of 4.0 mm. It was cut off straight and a sintered disk (porosity 1) was sealed in flush with the bottom of the tube. The top had a B7 socket with hooks. The tube was packed with resin, the total height of the resin being 2.2 ± 0.1 cm.

Fraction collector. A photoelectric drop-counting fraction collector (Locarte Co., 24 Emperors Gate, London, S.W. 7), capable of collecting 160 fractions in 6 in. $\times$ $\frac{1}{2}$ in. test tubes, was used. Drops formed at the square-cut, lower end (external diam. 3.2 mm.) of a small glass funnel, attached by a Terry clip to a rigid brass plate, which itself was attached by similar clips to the two rods supporting the photocell. The position of the bottom of the funnel relative to the photocell was adjusted in two directions by sliding the clips, until the growth of the small drops reduced the

photocell current as far as possible below its standing, maximum, uninterrupted value. This adjustment was facilitated by a microammeter (full scale deflexion $500\mu\text{A}$ and with shorting switch) permanently fitted in series with the photocell. Fractions of 0.2 ml. were obtained by collecting 8 drops of buffer containing detergent. The tip of the funnel was approx. 2 cm. above the top of the test tubes and was protected from draughts. Little evaporation from the fractions occurred overnight and no significant amount of ammonia was absorbed except in the neighbourhood of animal rooms. The funnel was kept permanently in the optimum position on the fraction collector and the blob at the bottom of the column brought gently into contact with the inside of the funnel at the beginning of an experiment.

Modifications to spectrophotometer. A Uvispek spectrophotometer (Hilger & Watts Ltd., model H 700.308) was temporarily modified as follows, so that readings could be made on 1.6 ml. of soln. in a standard 2 cm. cell. The cell was raised on a metal block 25 mm. $\times$ 13.5 mm. and 5.3 mm. in height, placed in the cell carrier. A diaphragm, consisting of a rectangular hole 4.5 mm. high and 1.8 mm. wide cut in a disk of black photographic paper, cemented to the end of a blackened metal tube (outside diam $\frac{3}{8}$ in. and inside diam. $\frac{1}{8}$ in. $\times$ 0.65 in. long), was inserted as close as possible to the exit slit, to obstruct the upper part of the light-beam. A slit width of 0.05 mm. and an input sensitivity of XI were used throughout with the meter sensitivity control set to position 4 for readings up to an extinction of 1.0, above which the meter sensitivity was increased as necessary.

METHODS

Operation of the columns

The columns were run as described by Eastoe (1955) except for the elution with pH 4.25 buffer being at 65° instead of 60° . After use, columns were cleaned with 0.2N-NaOH containing detergent. They were stored with alkali, the portion of the tube below the sinter being filled with water and closed with rubber tubing and clip while the top was stoppered. Where 1 mg. (or more) protein was available for analysis, separate (0.5 mg.) portions of hydrolysate were transferred to 100 and 15 cm. columns for determination of non-basic and basic amino acids respectively. Where less protein was available, a preliminary separation of basic amino acids was made on a column (2.2 cm. $\times$ 0.4 cm.) of Dowex 50. The hydrolysate in 0.2 ml. of citrate buffer, adjusted to pH 4.5–5.0, was added to this small column previously equilibrated with 0.1M-sodium citrate buffer, pH 5, and the eluate was collected in a conical graduated centrifuge tube. When all the hydrolysate had run into the resin, the column was eluted with more buffer, pH 5.0, in small (approx. 0.2 ml.) portions added as required. The elution was stopped when 1.3 ml. of eluate had collected. The eluate, which had been shown in preliminary experiments to contain all the

acidic and neutral amino acids, was adjusted to pH 2.5 by adding 0.15 ml. of 2N-HCl, and transferred to the 100 cm. column after 5 days' storage at -15° , during part of which period analysis of the basic amino acids was carried out, there being only one fraction collector available.

Basic amino acids were quantitatively retained on the small column. When the hydrolysate does not contain hydroxylysine, the resin may be completely transferred by means of a Pasteur pipette to the top of a column (15 cm. $\times$ 0.4 cm.), previously equilibrated with buffer, pH 5. The elution is then carried out with the usual buffer sequence (Moore & Stein, 1951). Inversion and mixing of resin layers, resulting from the above operation, prevents the separation of hydroxylysine from histidine.

When hydroxylysine may be present, the small column is mounted above the 15 cm. column by means of a rubber bung (Fig. 1) and the two are

Fig. 1. Method of mounting a column (2.2 cm. $\times$ 0.4 cm.) of Dowex 50 above a column (15 cm. $\times$ 0.4 cm.) for elution of basic amino acids. Capillary contact is maintained at point A and free liquid remains above resin in lower column throughout the elution.

eluted as a single unit. The small column and bung should be inserted very carefully and slowly into the top of the 15 cm. column, to prevent increase of air pressure forcing the resin in the small column upwards, away from the sintered disk. One cm. of liquid should remain above the resin in the lower column to allow for rise of room temperature during the experiment. Otherwise the enclosed air may expand sufficiently for it to enter the resin below and stop the flow of liquid. Mixing, in the small volume of liquid between the two portions of resin, does not cause significant loss of resolution. Adequate separation of hydroxylysine and histidine with 0.1M-sodium phosphate buffer, pH 6.75 (Eastoe, 1955), was obtained by this modification (Fig. 2).

It is necessary to elute for approx. 15% more fractions with each buffer to allow for the slight increase in the depth of resin. Early fractions, eluted with buffer, pH 5, should give no ninhydrin colour, serving to check the completeness of separation of non-basic amino acids.

Analysis of fractions

The volume of representative fractions was checked by weighing. All fractions were then adjusted to pH 5.0 by the addition of a definite number of drops of standard alkali or acid from calibrated jets, attached to glass reservoirs by short lengths of plastic tubing, each fitted with a screw clip. The volumes added are summarized in Table 1. Small corrections were made in the final calculations, where the fraction volume differed from 0.20 ml., and for the added drops. Portions (0.4 ml.) of ninhydrin solution (Moore & Stein, 1948) were added to the fractions from an automatic syringe pipette and the solutions were mixed by shaking the tubes. The tubes were covered with Oxoid aluminium caps (diam. $\frac{3}{4}$ in.) and heated for 20 min. in a boiling-water bath. The tubes were allowed to cool for 10 min., 1 ml. of ethanol-water (1:1, v/v) was added to each, and the solutions were mixed by shaking. The solutions were transferred to 2 or 0.5 cm. cells and the extinctions measured at 570 or 440 m μ in the spectrophotometer. Great care was necessary not to contaminate the solutions from the operator's fingers.

Fig. 2. Elution curve of the hydrolysate from 0.47 mg. of microsomal collagen, chromatographed on columns (2.2 cm. $\times$ 0.4 cm. and 15 cm. $\times$ 0.4 cm.) of Dowex 50 eluted as a single unit to separate the basic amino acids. The buffer was changed from 0.1M-sodium citrate, pH 5, to 0.1M-sodium phosphate, pH 6.75, at the point marked X, and finally to 0.2M-sodium citrate, pH 6.5, at the point marked Y. Note the absence of neutral amino acids in fractions 5-20.

Fig. 3. Elution curve of the hydrolysate from 0.47 mg. of microsomal collagen, chromatographed on a column (100 cm. $\times$ 0.4 cm.) of Dowex 50 after initial separation of basic amino acids on a column (2.2 cm. $\times$ 0.4 cm.). —, $E_{570\text{ m}\mu}$ with 2 cm. optical path; ---, $E_{570\text{ m}\mu}$ with 0.5 cm. optical path; ..., $E_{440\text{ m}\mu}$ with 2 cm. optical path for imino acids. The buffer was changed from pH 3.42 to pH 4.25 and the temperature from 37.5° to 65° at the point marked Z. Met.Sx, Methionine sulphoxide.

Table 1. Volumes of solution required for neutralization of fractions

All the buffers and both neutralizing solutions contained 0.4% (w/v) of BRIJ 35 to reduce surface tension.

Column	Buffer	Neutralizing soln.	Volume added (ml.)	No. of drops	Outside diam. of jet (in.)
100 cm. $\times$ 0.4 cm.	pH 3.42, 0.1M-sodium citrate	N-NaOH	0.024	2	0.045
	pH 4.25, 0.1M-sodium citrate	N-NaOH	0.012	1	0.045
15 cm. $\times$ 0.4 cm.	pH 6.75, 0.1M-sodium phosphate	0.5N-HCl	0.018	1	0.083
	pH 6.5, 0.2M-sodium citrate	0.5N-HCl	0.054	3	0.083

Determination of hydroxyproline. Hydroxyproline was determined by the method of Neuman & Logan (1950) as modified by Leach (1960). The determination was carried out in 6 in. $\times$ $\frac{3}{8}$ in. test tubes and the sample volume reduced from 1 ml. to 0.2 ml. with 1, 2 and 3 μ g. standards. All reagent volumes were reduced to one-fifth of those specified, solutions being measured with automatic zero, blow-out micropipettes (H. J. Elliott Ltd.). Reproducibility was better than on the larger scale.

Resolution and accuracy of the semi-micro-chromatographic method

The ninhydrin colorimetric method (Moore & Stein, 1948) when tested with 0.2 ml. portions of standard glycine solution gave a mean calibration factor of 0.0380 μ mole/unit of extinction for the 2 cm. light-path. This corresponded exactly with the value 0.190 previously obtained for 1 ml. fractions and a final volume of 8 ml. instead of 1.6 ml. The agreement suggested that colour formation was unaffected by the larger surface to volume ratio of the heated solutions on the smaller scale. Fading tests on the cold, diluted solutions gave erratic results but the fading rate averaged approx. 4%/hr., double that for the standard technique

(Eastoe, 1955). It is therefore advisable to complete extinction measurements within 40 min. of the end of the heating period.

The resolution of the various peaks is illustrated in Figs. 2 and 3, showing the elution curve of the hydrolysate from 0.47 mg. of microsomal neutral-salt-soluble collagen on columns 0.4 cm. in diameter. This compares well with results obtained on columns 0.9 cm. in diameter with 5–10 mg. loadings (see Eastoe, 1955, Figs. 1 and 2). These results indicate that variation of the diameter of narrow columns does not greatly influence the positions and sharpness of peaks, and suggest that the diameter could be reduced if necessary below 0.4 cm.

Owing to the doubtful purity of some amino acids available from sources in this country (Bender *et al.* 1959), the accuracy of the semi-micro method was investigated by comparing results obtained for a standard protein hydrolysate (acid-processed pig-skin gelatin, hydrolysed with 6N-HCl for 48 hr. at 100°) by using columns of 0.9 and 0.4 cm. diameter (Table 2). On the whole the two methods show as close agreement as can be expected from the known errors of colorimetry, and the differences do not greatly exceed those of duplicate analyses on a single column 0.9 cm. in

Table 2. Comparison of amino acid values for a hydrolysate of acid-processed, pig-skin gelatin with unmodified and semi-micro resin chromatographic methods

Values are given as g. of amino acid/100 g. of dry ash-free protein. For abbreviations of amino acids see *Biochem. J.* (1957), 66, 6.

Column diameter	...	0.9 cm.	0.4 cm.	
			Separate portions of hydrolysate on 100 and 15 cm. columns	Single portion separated on 2.2 cm. column
Ala		10.6	10.6	10.6
Gly		26.4	26.3	26.0
Val		3.24	3.01	3.25
Leu		3.36	3.50	3.54
Ileu		1.33	1.46	1.56
Pro		16.2	15.4	15.8
Phe		2.4	2.49	2.51
Tyr		0.51	0.46	0.45
Ser		3.89	4.09	3.86
Thr		2.27	2.32	2.24
Met		0.57	0.76	0.56
Arg		9.1	9.5	9.5
His		0.67	0.79	*
Lys		4.15	4.35	4.21
Asp		6.5	6.5	6.3
Glu		11.3	12.0	11.5
Hypro		12.7	(12.7)	(12.7)
Hylys		1.13	1.08	*
Total		116.32	117.31	115.96
Amide N		0.62	0.60	0.61
Wt. recovery (%)		97.2	98.0	97.0
N recovery (%)		101.5	102.5	101.4
Column loading (mg.)		5.8, 11.6	0.358 ($\times 2$)	0.358

* Sum of histidine and hydroxylysine 1.38.

diameter (Eastoe, 1955). The overall error with the semi-micro method is approximately $\pm 5\%$ for the larger peaks, compared with $\pm 3\%$ claimed for the original method (Moore & Stein, 1951). The increased error arises from the greater effect of variations in volume of test solutions and reagents together with fading. Errors for very small peaks may greatly exceed 5% , being mainly associated with assessing the base-line.

SUMMARY

1. A semi-micro modification of the Moore & Stein ion-exchange method of amino acid determination is described, which enables a complete analysis to be carried out on 0.3–0.6 mg. of protein.

2. The increased sensitivity obtained results from a reduction of resin-column diameter from 0.9 to 0.4 cm. with a corresponding reduction of the original volume of the fractions and reagents to one-fifth.

3. A simple method of modifying a spectrophotometer for convenient measurements on small volumes of solution in standard optical cells is described.

4. Preliminary separation of basic amino acids may be carried out on a column (2.2 cm. $\times$ 0.4 cm.) of Dowex 50 at pH 5.0. Separation of hydroxylysine from histidine is possible by combining the

small column with a 15 cm. column and eluting the two in series.

5. Colorimetry on the reduced scale is closely similar to the original procedure, but the rate of fading is increased.

6. The sharpness and resolution of amino acid peaks were not affected by reduction of column diameter.

7. A comparison of amino acid values for a gelatin hydrolysate analysed by the original and semi-micro methods showed good agreement. The overall accuracy of the modified method is approximately 5% .

I should like to thank Mr J. H. Trendall for his help in making the chromatography tubes, and the British Empire Cancer Campaign for providing a spectrophotometer.

This investigation was carried out during the tenure of a Senior Leverhulme Fellowship in Biochemistry.

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Some Properties of Coupled Iron–Caeruloplasmin Oxidation Systems

By G. CURZON

Department of Chemical Pathology, Institute of Neurology, The National Hospital, Queen Square, London, W.C. 1

(Received 19 October 1960)

Caeruloplasmin, the blue copper-containing protein of plasma, has been reported to have oxidase activity *in vitro* towards many substrates. Holmberg & Laurell (1951) found activity towards *p*-phenylenediamine, quinol, catechol, pyrogallol, dihydroxyphenylalanine, adrenaline and ascorbic acid. They used purified caeruloplasmin in the presence of albumin or gelatin, which tend to eliminate any effects due to trace metals. Abood, Gibbs & Gibbs (1957), Porter, Titus, Sanders & Smith (1957), Martin, Eriksen & Benditt (1958), Sankar (1959), Nakajima (1959), Curzon & Vallet (1960) and Levine (1960) found that 5-hydroxytryptamine and some related hydroxyindoles were oxidized *in vitro* by caeruloplasmin. It is not

known to what extent caeruloplasmin is involved in the metabolism of any of these substances *in vivo*.

During investigation of the effects of inorganic ions on the oxidase activity of purified human caeruloplasmin towards *NN*-dimethyl-*p*-phenylenediamine, it was found that the system was very sensitive to metals, both inhibition and activation being observed (Curzon, 1960). The most marked activating effect was found with ferrous iron. Curzon & O'Reilly (1960) showed that caeruloplasmin was able to oxidize ferrous to ferric iron and explained the activating effect of iron in terms of a coupled iron–caeruloplasmin oxidation system. This paper describes some properties of the system