

APPENDIX

Notes on the Determination of Amino Acids by Ion-exchange Chromatography

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Moore & Stein (1951) introduced a method for the quantitative determination of amino acids by separation on Dowex 50 columns, eluted with buffer solutions, followed by analysis of the eluent fractions with a colorimetric ninhydrin method (Moore & Stein, 1948). Certain details of their procedure were slightly modified in the present investigation as described below.

Materials

Dowex 50. The resin, kindly supplied by Dow Chemical International Ltd., Midland, Michigan, U.S.A., was stated to be of nominal 12% cross-linking and 200-400 mesh.

Sodium phosphate buffer pH 6.8, 0.1M. This buffer was adjusted to pH 6.75 in order to delay slightly the emergence of the histidine peak. At the lower pH, histidine emerges separated from and almost mid-way between hydroxylysine and lysine on the 15 cm. column.

Thymol. The concentration of thymol incorporated in the buffer solutions was controlled at 0.25 g./l. Solutions kept for two years at room temperature showed no signs of mould growth. Thymol did not significantly alter the pH of the solutions but markedly affected the surface tension, even when detergent was present.

BRIJ 35. A 33% (w/w) solution of BRIJ 35 (Honeywill and Stein Ltd., 21 St James's Square, London, S.W. 1) was freshly prepared each week, as some samples of detergent were contaminated with mould spores.

Thiodiglycol. Technical-grade thiodiglycol (L. Light and Co. Ltd., Colnbrook, Bucks.) was freed from an impurity which gave a yellow colour with ninhydrin by the method described previously (Eastoe & Eastoe, 1954).

Ethylene glycol monomethyl ether. 'Methyl oxitol' (Honeywill and Stein Ltd.) was redistilled to remove ninhydrin-positive impurities. To minimize risk of explosion due to decomposition of peroxides, 10 ml. of a solution of 30 g. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 3 ml. of H_2SO_4 in 55 ml. of H_2O were added per litre before distillation and the final 10% of the solvent was left undistilled and discarded.

Ninhydrin. Ordinary-grade ninhydrin (British Drug Houses Ltd.) was purified in 87% yield by the method of Moore & Stein (1948). The ninhydrin-stannous chloride reagent was prepared freshly each week, since the reagent, which was stored in the dark under nitrogen, showed signs of change after 2 weeks in hot weather. No significant change in the colour intensity produced on heating with amino acids could be observed during the first week after preparation.

Diluent solution. Equal volumes of water and absolute ethanol were mixed (Moore & Stein, 1954).

Fraction collector. The fraction collector used in this study was built in the laboratory and worked on the drop-

counting principle, a fixed number of drops (20) being collected in each fraction after the operation of a capacity switch, thyatron relay and electromagnetic counter.

Operation of the columns

The elution was started with citrate buffer (pH 3.42) and a temperature of 37.5°, as used by Moore & Stein (1951). At the beginning of the emergence of the valine peak, the buffer was changed to one of pH 4.25 and the temperature raised to 60°, where it was maintained until after the emergence of phenylalanine, when the chromatogram was terminated. This procedure was preferred to the one of Moore & Stein (1951), where the temperature was raised first to 50° at the buffer change and finally to 75° immediately after the emergence of isoleucine. This latter occurred during the night, since the chromatograms were normally started during the morning and was therefore inconvenient. The intermediate temperature enabled a similar separation of tyrosine and phenylalanine to that of the original sequence to be achieved, without spoiling the separation of isoleucine and leucine. Basic amino acids were removed from the column with 100 ml. of 0.2N-NaOH. The chromatogram was run at 4.5 ml./hr. for approximately 48 hr. before the buffer was changed and the temperature increased. The chromatogram was completed in a further 30 hr. at a somewhat greater rate of flow at the higher temperature. Columns have been in use for 18 months without need for repacking. The flow rate for a given head decreased as the resin packed down during the first few chromatograms and then remained constant.

The 15 cm. \times 0.9 cm. columns were eluted as described by Moore & Stein (1951), except that the first buffer change was made at fraction 25 instead of fraction 20, in order to avoid a sharp rise in the ninhydrin blank when the phosphate buffer emerged. The slight lowering of the pH of the phosphate buffer to separate histidine from hydroxylysine has been mentioned above.

Analysis of the fractions

The optical densities of the coloured solutions were measured in optical cells with a Uvispek spectrophotometer at 570 m μ . Since linear readings were obtainable up to an optical density of at least 1.5, it was possible to measure all fractions, except those at the top of the glycine peak, with only 5 ml. of diluent solution. All fractions were measured against water and the appropriate blank values subtracted later.

Effect of variations in technique on the colour intensity

The rate of fading of the cold diluted solutions was found to be approximately 1½-2%/hr. and took place from the beginning. Moore & Stein (1948) found no fading within the

first hour after cooling, followed by fading at 1% per hr. The present experiments were made in the absence of thiodiglycol, and this may account for the different results. The rate of fading was slightly increased for quantities of amino acid below 0.05 μ mole.

The temperature of the coloured solution at which the optical density was measured was found to affect the reading. There was a 2% decrease in colour for a 10° rise in temperature, the effect being reversible. This emphasized the need for a standardized and sufficiently long period of cooling before dilution.

Calculation of tyrosine content

The tyrosine and phenylalanine peaks showed a considerable overlap, which made the estimation of tyrosine difficult, especially where only a small quantity was present. Since the overlap extended as far as the top of the tyrosine peak, the method of calculation of Stein & Moore (1948) was inapplicable, as it gave erroneously high values for tyrosine, the height of the peak being augmented by the toe of the phenylalanine peak.

It was found that a reasonably good fit of all except the most asymmetrical amino acid peaks was given by the equation

$$y = A(1 + Bx^2) e^{-x^2/C^2},$$

where the fraction number x is zero when y , the optical density, is a maximum, and A , B , C are constants (Ward, private communication). A is equal to the maximum value of y , since $x = 0$. This equation was applied to the phenylalanine peak and the values of the constants were calculated by inserting experimental values of y for two points $\pm x$ on either side of the summit, as far away from the top as was judged to be safe from the point of view of overlap with the tail of the tyrosine peak. The shape of the toe of the phenylalanine peak was then calculated by inserting suitable values of x in the equation, and hence, by difference from the experimental data, the points on the tyrosine peak. This method gave two peaks similar in shape to those of the isolated peaks for other amino acids, the tyrosine peak being nearly symmetrical, but it was considered too tedious for routine application.

An empirical method was developed by selecting points close to the first maximum or second point of inflexion of the experimental curve (assuming them to be the maxima of the tyrosine peak) and constructing the falling portion of the tyrosine peak (assuming that it is symmetrical and that the over-

lap of phenylalanine was negligible before the selected maximum). From each of the several tyrosine peaks plotted, the shape of the toe of the phenylalanine curve was calculated by difference. The shape nearest to that usually obtained experimentally for other peaks was readily selected and the corresponding values for tyrosine and phenylalanine were calculated. The results were in good agreement with those deduced by application of the equation for the peak.

SUMMARY

1. A temperature of 60° is employed throughout the elution of the 100 cm. Dowex 50 column with sodium citrate buffer (pH 4.25), instead of a change from 50 to 75° after the emergence of isoleucine.
2. The 0.1M sodium phosphate buffer used to elute the 15 cm. Dowex 50 column was reduced from pH 6.80 to 6.75 to separate histidine from both hydroxylysine and lysine.
3. The optical density of the coloured solutions was measured in optical cells instead of in test tubes. This enabled a sensitive spectrophotometer having a linear scale up to at least 1.5 optical density units to be used, leading to increased accuracy and convenience in avoiding frequent dilution of samples.
4. An empirical method for calculating tyrosine values is described which is applicable when there is considerable overlap by phenylalanine and only a small quantity of tyrosine present.

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