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An Improved Analytical Procedure for Automatic Analysis of Amino Acids

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There is an increasing need for rapid and precise determination of amino acids. Hamilton (1960), and Hamilton, Bogue & Anderson (1960) have identified the critical factors affecting the chromatographic separation of amino acids. By using beads of small and uniform size, narrow-bore columns and an eluting gradient it is now possible to separate all the amino acids in a protein hydrolysate. Column resolution is so sharp that many of the amino acids are eluted as peaks of no more than 1 min. duration.

High resolution is a product of both chromatography and analysis, and though much attention has been paid to column resolving power there has been comparatively little development of the analytical system. In the automated analytical system most commonly used, the column effluent is mixed with the reagent (ninhydrin/hydrindantin 10% methylCellosolve heavily buffered) and heated at 95° in a long-time-delay coil to allow for colour development (Piez & Morris, 1960; Spackman & Moore, 1958). Diffusion is minimized by using a long capillary coil or by segmenting the coil with oxygen-free nitrogen. We have used a new method with the Technicon Autoanalyzer. In this system a steady concentration of an amino acid is maintained throughout this analytical system, we have found that 5 min. is required for the full colour to be attained and over 1 min. for the half-maximum value to be reached. There is an equilibrium on instantaneous withdrawal of the effluent. This slow rise time seriously impaired

the overall resolution of the chromatogram. We attribute this slow rise time to the presence of methylCellosolve, since similar wholly aqueous systems have a much more rapid response. A slow rise time of this kind will decrease the resolution of closely adjacent peaks even on a slow (22 hr.) chromatogram. A further disadvantage of analytical procedures that use hydrindantin is the extreme sensitivity to oxygen. The reagent is therefore difficult to prepare and not easily stored. It also necessitates the rigid exclusion of air from the entire system, a condition not easy to satisfy adequately when using gradient elution, particularly with the high pumping pressures required with fast chromatograms. We have repeatedly found a random variation in colour yield in different parts of the chromatogram. This we attribute to air dissolved in the buffers at the high pressures used. A high background colour is also a significant feature.

It would be clearly desirable to have an analytical system that was insensitive to oxygen and containing little or no methylCellosolve and consequently showing a faster rise time. We experimented with the trinitrobenzenesulphonic acid method suggested by Hochstrasser (1965), but in addition to being insensitive to proline and hydroxyproline, which have to be determined separately, the colour yield was only about one-fifth of that of the ninhydrin system. Cadavid & Paladini (1964) described a ninhydrin method adapted from that of Rosen, Berard & Levenson (1962) in which added hydrin-

is replaced by sodium cyanide. The sodium reduces some of the ninhydrin to form hydantoin during the determination. This procedure gives colour yields comparable with those obtained with hydriindantin. The colour development obeys Beer's law and the reaction is sensitive to oxygen.

We have modified this method to give several important advantages. The flow diagram is shown in Figure 1. The replacement of hydriindantin in aqueous 0.2M-sodium cyanide introduced into the system separately permits the decrease of ethylCellosolve concentration to 20% of its original value with no danger of crystallization of hydantoin in the system. This makes a substantial improvement in the rise time together with a considerable gain in overall resolution. This system is insensitive to oxygen and in fact allows aeration of the reagent stream with air. However, it gives a uniformity of colour yield throughout the chromatogram, a feature that could not be achieved previously. The reagents are easy to make up reproducibly and are stable for long periods. The background colour is extremely low (0.08). The colour development follows Beer's law.

The chromatographic system comprises five columns (75 cm. $\times$ 0.6 cm. internal diam.) jacketed and packed with Chromobeads B (Technicon Instruments Co.). Buffer from a nine-chambered Autograd is pumped through each column by a Roy Minipump at a flow rate of 1 ml./min. The columns are run sequentially, permitting analyses to be completed in 24 hr. The complete system includes data-processing facilities (Evans, Axford, Siriwardene & Robins, 1965; Robins, R. A. Evans, J. A. de S. Siriwardene & Thomas, unpublished work). The eluting buffers are: 1, buffer, pH 2.75, containing methanol (0.5 v/v), thiodiglycol (0.5%) and Na^+ (0.2M); 2, buffer, pH 2.75, containing thiodiglycol (0.5%) and Na^+ (0.2M); 3, buffer, pH 2.90, containing thiodiglycol (0.5%) and Na^+ (0.2M); 4, buffer, pH 10.0, containing Na^+ (0.8M); 5, buffer, pH 10.0, containing Na^+ (2.2M).

All buffers are 0.05M with respect to citrate and contain BRIJ 35 (1%, v/v). The Autograd composition is as follows: chamber 1, 32 ml. of buffer 1; chamber 2, 32 ml. of buffer 2; chambers 3, 4 and 5, 32 ml. each of buffer 3; chambers 6 and 7, 32 ml. each of buffer 4; chambers 8 and 9, 32 ml. each of buffer 5.

Results. All the amino acids except for serine/threonine, glutamic acid/proline, isoleucine/leucine and tyrosine/phenylalanine are completely resolved and the troughs between these pairs are less than one-third of the peak height for equimolar mixtures. Accurate calculation of results is thus possible in all but extreme cases. A more flexible gradient-elution system is under development which may improve separation of all incompletely resolved amino acids.

Reproducibility of results with this system is good. For eight replicate samples of a casein hydrolysate, the mean coefficient of variation for all the amino acids was 3.6. The largest variation occurred with aspartic acid and alanine, with coefficients of variation 6.7 and 7.4 respectively. The chromatogram trace suggests that this high variation could be due to small amounts of interfering material.

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