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Note

Fully-automated gradient-elution system for amino acid analysis

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Gradient elution was first applied to amino acid analysis by Piez and Morris[1], and Thomas[2] has subsequently described the construction of an automated gradient former as part of a fully-automated amino acid analyser. Tschesche *et al.*[3] designed a semi-automated system using a commercially available gradient former (Ultrograd, LKB, Stockholm, Sweden) and the present paper describes the incorporation of the Ultrograd into a fully-automated single-column system which analyses one protein hydrolysate per 4 h. The complete instrument is shown diagramatically in Fig. 1 and the operation of the gradient-elution and sample-loading systems are described.

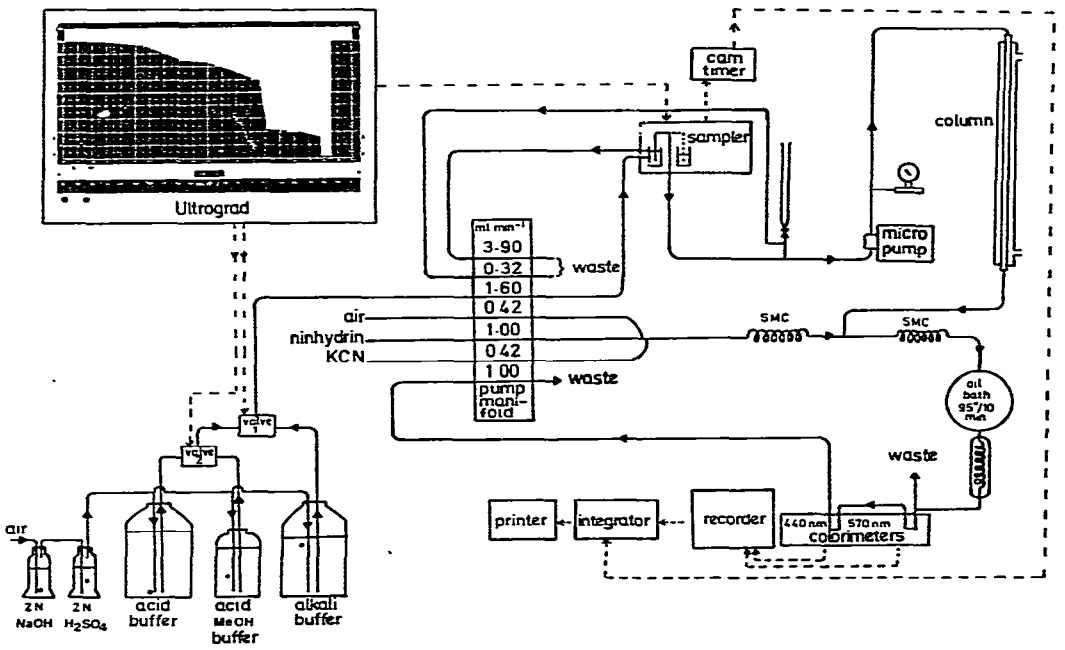

Fig. 1. Diagram of fully-automated amino acid analyser, incorporating gradient-elution system. Solid lines represent liquid flow and broken lines electrical connections.

EXPERIMENTAL

Gradient system

The gradient buffers are based on those described by Stephens[4] and include boric acid to improve buffering capacity in the region pH 7–11. The method of preparation ensures a low baseline rise due to ammonia. In distilled water, 42 g citric acid, 32 g sodium hydroxide and 2.48 g boric acid are dissolved, the volume is made to about 3.6 l and the solution is boiled for 30 min. After cooling, 4-l batches of each buffer are made as follows. (i) Alkali buffer, 20 ml 20% Brij-35 are added and the volume made to 4 l. (ii) Acid buffer, 20 ml 20% Brij-35 and 20 ml thiodiglycol are added, the pH is adjusted to pH 2.2 with redistilled hydrochloric acid and the volume made to 4 l. (iii) Acid methanol buffer is made up as for (ii) but with the addition of 400 ml redistilled methanol before pH adjustment.

The gradient buffers are contained in large glass reservoirs (5 l for acid methanol and 20 l for acid and alkali). Air, entering these to replace the pumped-out buffer, is first scrubbed by passing through wash bottles containing 2 *N* sodium hydroxide and 2 *N* sulphuric acid and is then passed to the bottom of the reservoirs from where it bubbles through the buffer. This Mariotte-flask arrangement ensures that regardless of the level of buffers the effective heads remain equal in all three reservoirs. The outlets from the reservoirs are controlled by the Ultrograd gradient mixer using the solenoid valves arranged as shown in Fig. 1. With Ultrograd mode switch in position II and a scan time of 4 h the gradient consists of acid methanol + alkali buffer for the first 18 min of the run, after which valve 2 is switched by means of the index control and acid + alkali buffer are mixed for the remaining 222 min of the run.

The essential features of the gradient curve as shown in Fig. 1 are (i) an initial period during which the acid methanol + alkali are run; the duration of this is adjusted to give optimal resolution of threonine–serine and glutamic–proline, (ii) a gradually increasing pH to elute acid and neutral amino acids, (iii) a sharp increase in pH followed by a plateau to allow the elution of the basic amino acids, (iv) an alkali column wash, (v) regeneration of the column with buffer at the same pH as is used at the start of a run.

The buffer gradient is drawn through the solenoid valves and pumped into the wash cup of the sampler, using a channel of the proportioning pump. The in-line magnetic stirrer provided with the Ultrograd is not used, since sufficient mixing of the gradient occurs in the tubing and the sampler wash-cup. The size of the latter has been reduced from 10 to 0.3 ml to prevent excessive mixing of the gradient, which would result in a slower response to the pH changes dictated by the Ultrograd. The buffer gradient is drawn from the sampler wash cup via the sampler probe by the high-pressure micro-pump (Milton-Roy, Riviera Beach, Fla., U.S.A.) which delivers it to the column at 0.55 ml/min. Excess buffer runs into the sampler drain cup and is pumped to waste by the proportioning pump. Air is prevented from entering the micro-pump by a de-bubbler connected in line and through which a proportion of the gradient is continuously pumped to waste by the proportioning pump. This ensures that no air can enter the micro-pump even when sampling occurs. A flow meter is attached to the de-bubbler as shown (Fig. 1) and is used to check the flow-rate as required. The column (750 × 6 mm, Technicon) is contained in a water jacket at 60°

and is packed with a 11–15 μm spherical-bead cation-exchange resin (Aminex A-5, Bio-Rad Labs., Bromley, Great Britain); the column back-pressure (*ca.* 20 kg/cm^2) is monitored by a diaphragm pressure gauge.

Sample loading

The sampler (Model A40, Hook and Tucker, London, Great Britain) has been modified by the makers so that it will operate on receipt of an external signal. The Ultrograd has two power sockets which can be used for running a pump; the power to this ceases during the reset period at the completion of the gradient. One of these sockets is connected to provide the external signal to the sampler. The sample time generally used is 40 sec and hence, at a pump rate of 0.55 ml/min and the sample dilutions employed, about 0.1 μM norleucine is loaded per run. Standards and samples are prepared in 0.1 N HCl; variations in sample time to allow for differences in concentration, do not impair the resolution. Sampler wash time is set so that the total sampler cycle period exceeds 120 sec which is the reset time of the Ultrograd and this ensures that only one sample is loaded per run. The Ultrograd is set up to automatically produce another gradient after resetting.

Peak detection and integration

An air-stable ninhydrin system similar to that described by Thomas *et al.*[5] is used for colour development (Fig. 1). The signals from the two colorimeters are integrated electronically (CRS 210, Infotronics, Shannon, Ireland). The reset signal to the integrator at the end of a separation is delayed during the integration of the basic amino acid peaks by a cam timer which is actuated by the main-circuit relay of the sampler and which after a pre-set delay of 25 min sends reset and re-start signals to the integrator.

CONCLUSION

The analyser gives excellent resolution of protein hydrolysates with a total time per sample of 4 h. Although there have been considerable advances in amino acid methodology in the five years since its construction, improvements such as faster resins or a more sophisticated data-handling system could easily be incorporated into the system.

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