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SUBMICRO-DETERMINATION OF AMINO ACIDS BY A SINGLE-COLUMN ANALYSIS IN A 4-HOUR RUN USING AUTOMATED BUFFER GRADIENT MIXING

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SUMMARY

A pH gradient for the single-column submicro-analysis of all protein-constituent amino acids in a 4-h run is described. High sensitivity in the 10 nmole range is achieved by using smaller columns than usual with an I.D. of 4.5 mm. Automatic mixing of the pH gradient is achieved by means of an Ultrograd gradient mixer, which is operated by timers and a control unit in a 24-h cycle to give five analyses daily. The control unit can be easily assembled from spare parts. The circuit diagram is presented.

INTRODUCTION

The updating of available equipment for amino acid analysis is necessary because of the expense of the commercially available instruments and the rapidly increasing number of analyses that must be carried out daily. The development of automatic sequencers for the determination of protein structure[1,2] has effectively decreased the period necessary for sequence work on peptides and proteins. In order to keep up with the speed of structural studies involving degradations, it has become necessary to increase the amino acid analysis capacity in many laboratories. This demand for more analyses per day has even become more drastic since in many laboratories the acid hydrolysis of phenylthiohydantoins into the corresponding free amino acids[3] is used continuously for the identification of the amino acid derivatives obtained from the Edman degradation of polypeptide chains.

We already have described[4] a modification of a commercially available system (Technicon AutoAnalyzer I) for the single-column analysis of protein-constituent amino acids, according to Piez and Morris[5], on the submicro-scale using a 0.3 × 120 cm column and a 10.5-h gradient. In this paper, we report on a set of modifications that enable a chromatogram to be completed within 4 h using automated buffer gradient mixing. In addition, an automatic control unit is described that controls the operation of five analyses per day. Hence five analyses can be completed in a 24-h cycle.

EXPERIMENTAL

Analytical system

The instrument is a standard piece of apparatus (Technicon AutoAnalyzer I) except that it is equipped with micro-columns 0.45 × 80 cm made from precision-bore glass tubes (Fa. Schott, Mainz, G.F.R.; 4.5±0.1 mm I.D., 8.9±0.3 mm O.D. KPG tubes). The resin consists of Chromobeads B, in the form of 17±1 μm spherical particles to reduce the back-pressure. The columns are filled to a height of about 70 cm using the final operating pressure of 350–400 psi throughout the procedure. The operating temperature of the column is 61 ±0.2°. The developing buffer is pumped at an input flow-rate of 0.78 ml/min, which develops a pressure of about 350 psi in the column during a run. The column effluent flows directly into the nitrogen-segmented ninhydrin stream by means of a T-piece (Technicon H-3) fitted at the column outlet. Therefore, all of the resolved material eluted from the column is used for detection and is segmented immediately after leaving the column, thus preventing remixing and peak spreading. The segmented ninhydrin stream passes through all five chromatographic columns, which are developed one after another (Fig. 1). The pumping tubes for the proportioning pump lines are commercially available and deliver 0.42 ml/min of nitrogen and 1.69 ml/min of ninhydrin, while 1.20 ml/min is aspirated back through the lines of the flow cells of the colorimeters. The light path through the longitudinal-type flow cells of the 570 and 440 nm colorimeters is 15 mm. The flow time for ninhydrin colour development in the heating bath coil is *ca.* 13 min. The ninhydrin solution for colour development consists of 5 g of ninhydrin and 0.5 g of hydrindantin dissolved in 162 ml of distilled methyl cellosolve plus 87.5 ml of sodium acetate buffer of pH 5.5. Finally, the solution is diluted with 375 ml of methyl cellosolve and 375 ml of water. All other parts of the analytical system remain unchanged.

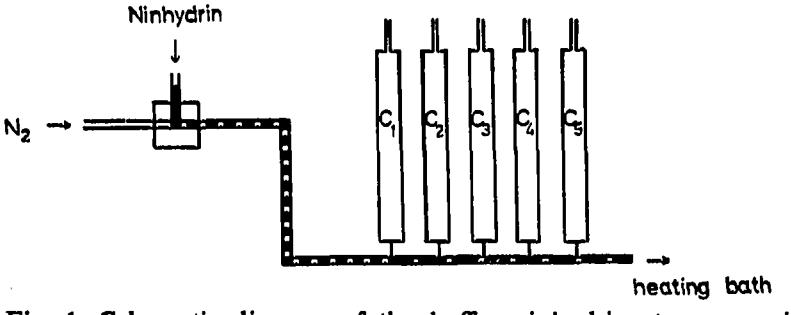

Fig. 1. Schematic diagram of the buffer–ninhydrin stream passing the column outlets of the chromatographic columns C_1–C_5 flowing into the heating bath.

The buffer gradient for elution of the acidic, neutral and basic amino acids is mixed by an automated buffer gradient mixer (LKB-Ultrograd, LKB, Stockholm, Sweden) from three different buffer reservoirs of pH 2.58, 3.80 and 12.00, according to Figs. 2 and 3. All buffers are prepared from a stock solution by careful adjustment of pH with 6 *N* HCl. The stock solution is made from a concentrate prepared from 110.32 g of sodium citrate and 167.5 ml of 2 *N* NaOH in water heated to boiling for 30 min in order to remove trace amounts of ammonia. Then 75 ml of Brij 35

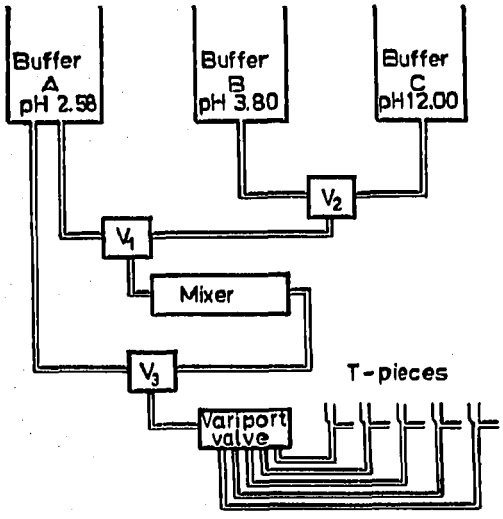

Fig. 2. Schematic diagram of the arrangement of the three buffer reservoirs A, B and C, the LKB valves V_1 and V_2, which are operated by the mixer, and the LKB valve V_3, which is operated by the timers at position 3. The buffer mixing system is connected to the individual chromatographic columns by means of the Variport valve. Each of the individual buffer lines to the Milton Roy pumps contains an air trap (T-piece).

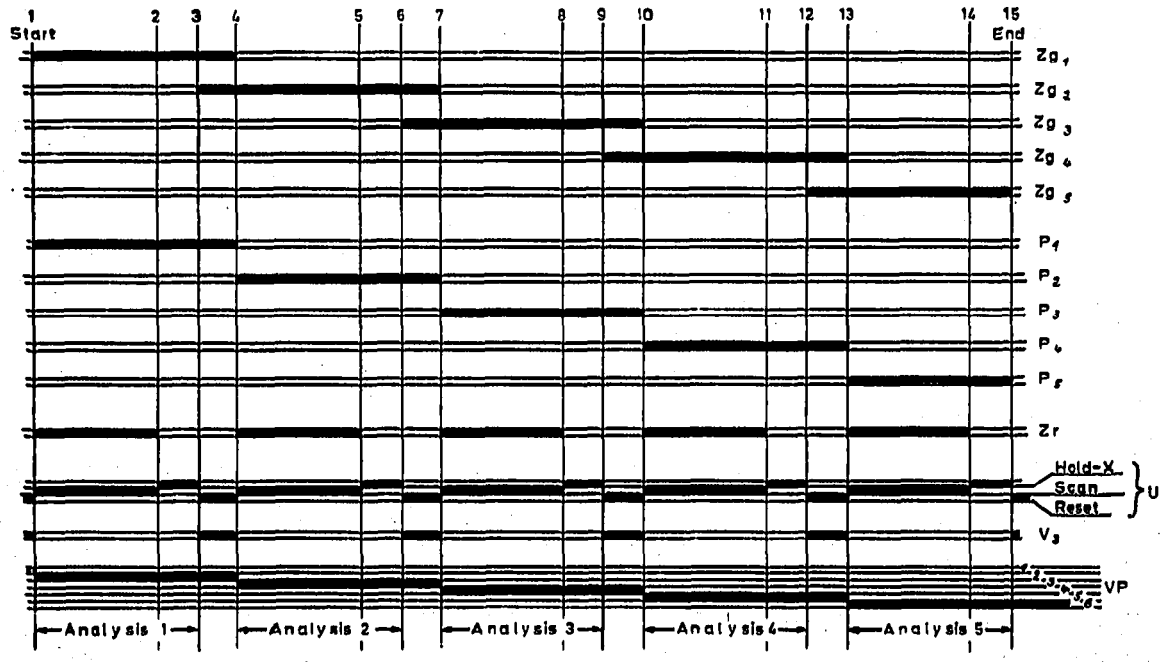

Fig. 3. Schematic representation of the operation and shut-off of the individual components. P, Milton Roy pumps; Zr, timer to give an impulse to the Ultrograd for "Hold-X"; U, preset operation of the Ultrograd; V_3, LKB valve operated by the timer at position 3; VP, Variport valve connecting the gradient mixer to the individual Milton Roy pumps.

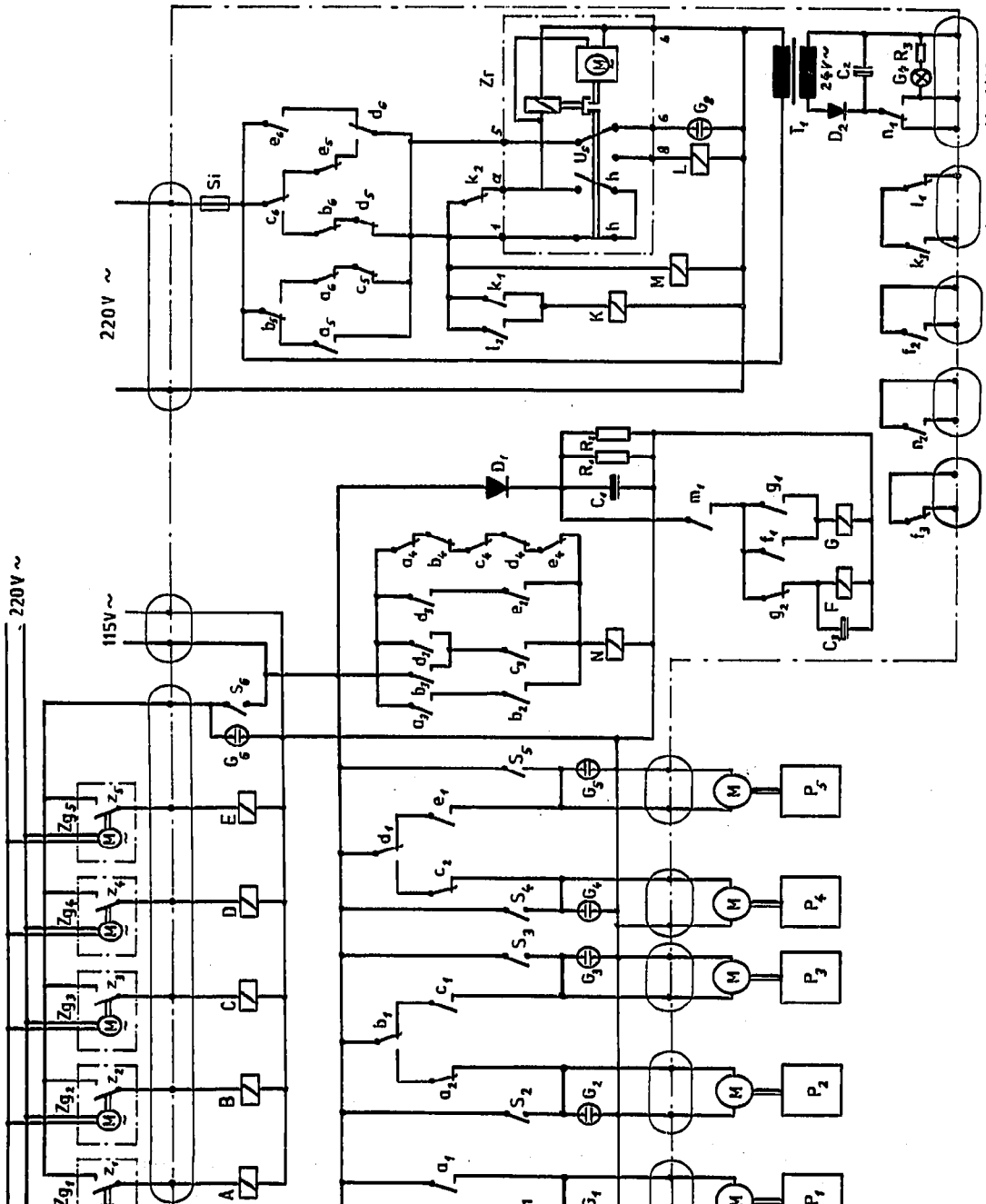

Fig. 4. Circuit diagram of the control unit. Zg_1–Zg_5, timers; A–E,N, A.C. relays (115 V); F, G, D.C. relays (115 V); K–M, A.C. relays (220 V); Zr, timer (220 V, 0.2–5 h); G_1–G_6, glow-lamps (115 V); G_7, lamp (24 V); G_8, glow-lamp (220 V); Si, fuse; S_1–S_6, toggle switches; C_1, 8 μF/350 V; C_2, 1000 μF/35 V; C_3, 10 μF/350 V; R_1, R_2, 10 kΩ/7 W; R_3, 22 Ω/1 W; D_1, BY 143; D_2, BY 116; P_1–P_5, Milton Roy pumps; T_1, transformer (220 V–24 V).

(1 g of poly(ethylene glycol)monolauryl ether in 3 ml of water) and 0.8 g of sodium azide are added and the mixture is diluted to a total volume of 7 l. The buffer reservoirs are filled with 4–6-l aliquots of this solution, carefully adjusted to the desired pH of 2.58, 3.80 or 12.00; the latter buffer contains 1.8 *M* NaCl.

The column is regenerated by pumping 0.2 *N* NaOH for 30 min and buffer adjusted to pH 2.91 for a further 15 sec. When not in use, the columns are kept under 0.2 *N* NaOH. This procedure should be followed in order to keep the ammonia base-line down and the base-line rise out of the phenylalanine peak.

After the column has been loaded with the sample for analysis, dissolved in 0.1–0.3 ml of distilled water (with 0.03 μmole of norleucine as internal standard), it is filled with buffer of pH 2.72. The buffer delivery lines from the Variport valve via the Milton Roy pump to the top of each column contain 10 ml of buffer of pH 2.72, which itself contains 12% of *n*-propanol in order to increase the resolution of threonine and serine. *n*-Propanol was found to be superior to methanol because of its higher boiling-point and its lower tendency to dissolve in the resin. Thus a continuous flow through the ninhydrin colour bath could be obtained, which resulted in an undisturbed base-line and permitted the determination of cysteic acid in all chromatograms.

Control unit

In order to complete five chromatograms one after another with the same gradient mixing device, a number of relays were connected in a switchgear unit as shown in Fig. 4. The operation of this unit is controlled by five timers (Sangamo timers, Technicon GmbH, Frankfurt, G.F.R.), which provide the impulses for operation and switch off the Milton Roy pumps, the Ultrograd gradient mixer, the LKB valve V_3 and the Variport switching valve (Serva Technik, Heidelberg, G.F.R.) that connects the gradient mixing system to the next column as soon as one chromatogram has been completed. The cost of the complete unit and accessories is low, as it can be easily assembled from commercially available spare parts.

The operation of the complete system, as directed by the control unit and timers, is demonstrated schematically in Figs. 2 and 3. The black bars in Fig. 3 indicate the operation of the various components: Zg_1–Zg_5, timers; P_1–P_5, Milton Roy pumps; Zr, timer making contact to stop the Ultrograd in position "Hold-X", giving buffer delivery of constant pH; and VP, Variport valve connecting the next column system to the Ultrograd gradient mixer. At position 1 in Fig. 3, the timer Zg_1 for column 1 starts the Milton Roy pump P_1. The same signal is used to start the programme of the Ultrograd U and the timer Zr. The amino acid analysis is started and continuously recorded. At position 2, the timer Zr gives a signal to the Ultrograd to stop scanning the programme in the "Hold-X" position in order to deliver buffer of constant composition. This arrangement facilitates more accurate adjustment of the Ultrograd programme and the elution time necessary to complete the amino acid chromatogram. At position 3, the timer Zg_2 (related to column system 2) gives a signal to prepare Milton Roy pump P_2 for operation, *i.e.*, as soon as timer Zg_1 switches off Milton Roy pump P_1 the pump P_2 is at its full operating power. This device is necessary so as to ensure the continuous flow of a constant stream through the analytical system. The impulse at position 3 is used to switch the connection of the Ultrograd gradient mixer and the Milton Roy pump P_1 to the

reservoir of the starting buffer R_4 (pH 2.85) by means of LKB valve V_3. This procedure ensures that all lines of the Variport valve and up to the Milton Roy pump P_2 are filled with the starting buffer for the next run (run 2). At position 4, the timer Zg_1 switches off, giving an impulse that is used to switch Milton Roy pump P_1 out of operation and simultaneously to start Milton Roy pump P_2. In addition, the Ultrograd gradient mixer is started again from its "Start" position to scan the gradient programme, as it has now been reset by the impulse at position 3. At the time when the new programme is started, LKB valve V_3 switches back to connect the gradient system to the Milton Roy pump P_2, disconnecting the buffer reservoir R_4. Analysis 2 is now in full operation. This procedure is repeated until all analyses 1–5 have been completed.

In order to allow manual operation, the timers can be switched off by the toggle switch S_6 (Fig. 4). Then the relays A–E are out of operation and the Milton Roy pumps can be started by the toggle switches S_1–S_5.

Operation of the Ultrograd gradient mixer is started at positions 1, 4, 7, 10 and 13 by operating the contacts Z_1–Z_5 (Fig. 3). Then the relay contact m_1 is closed and releases the monostable multivibrator circuit (relays F and G), which, on contact f_3, gives the starting impulse for the Ultrograd gradient mixer. The slow release of relay F is achieved by condenser C_3 preventing flutter of the contacts, which might fail to start the Ultrograd.

RESULTS AND DISCUSSION

The resolution of a typical chromatogram of a standard mixture of 0.02 μmole each of the protein-constituent amino acids is shown in Fig. 5. All amino acids are resolved and can easily be quantitated by integration of the individual peaks by any conventional method. Norleucine was added as an internal standard and appears immediately after isoleucine and leucine and before the aromatic amino acids. Tryptophan, not present in the mixture, gives a peak immediately after histidine and some time before arginine.

The pH gradient giving a resolution of all protein-constituent amino acids in the chromatogram is shown in Fig. 6 and can be prepared by means of a nine-chamber Autograd, as described previously[6]. It is possible, however, to prepare

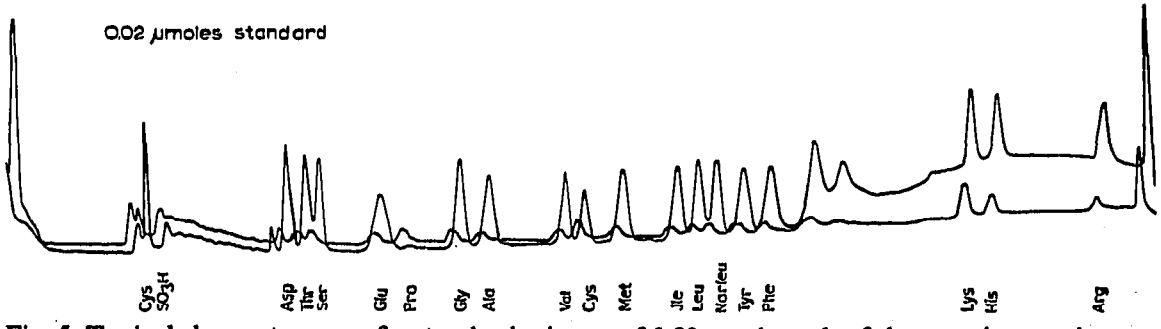

Fig. 5. Typical chromatogram of a standard mixture of 0.02 μmole each of the protein-constituent amino acids resolved in a single 4-h run by the gradient shown in Fig. 6. Region Asp to Arg. No range expansion.

the same gradient by automatic mixing of three buffers of pH 2.58, 3.80 and 12.00 (1.8 *M* NaCl) using an LKB Ultrograd gradient mixer. The Ultrograd mixer controls two three-way LKB valves (V_1 and V_2 in Fig. 2), according to a valve timing diagram, which is the gradient curve chart that is automatically scanned. The gradient curve

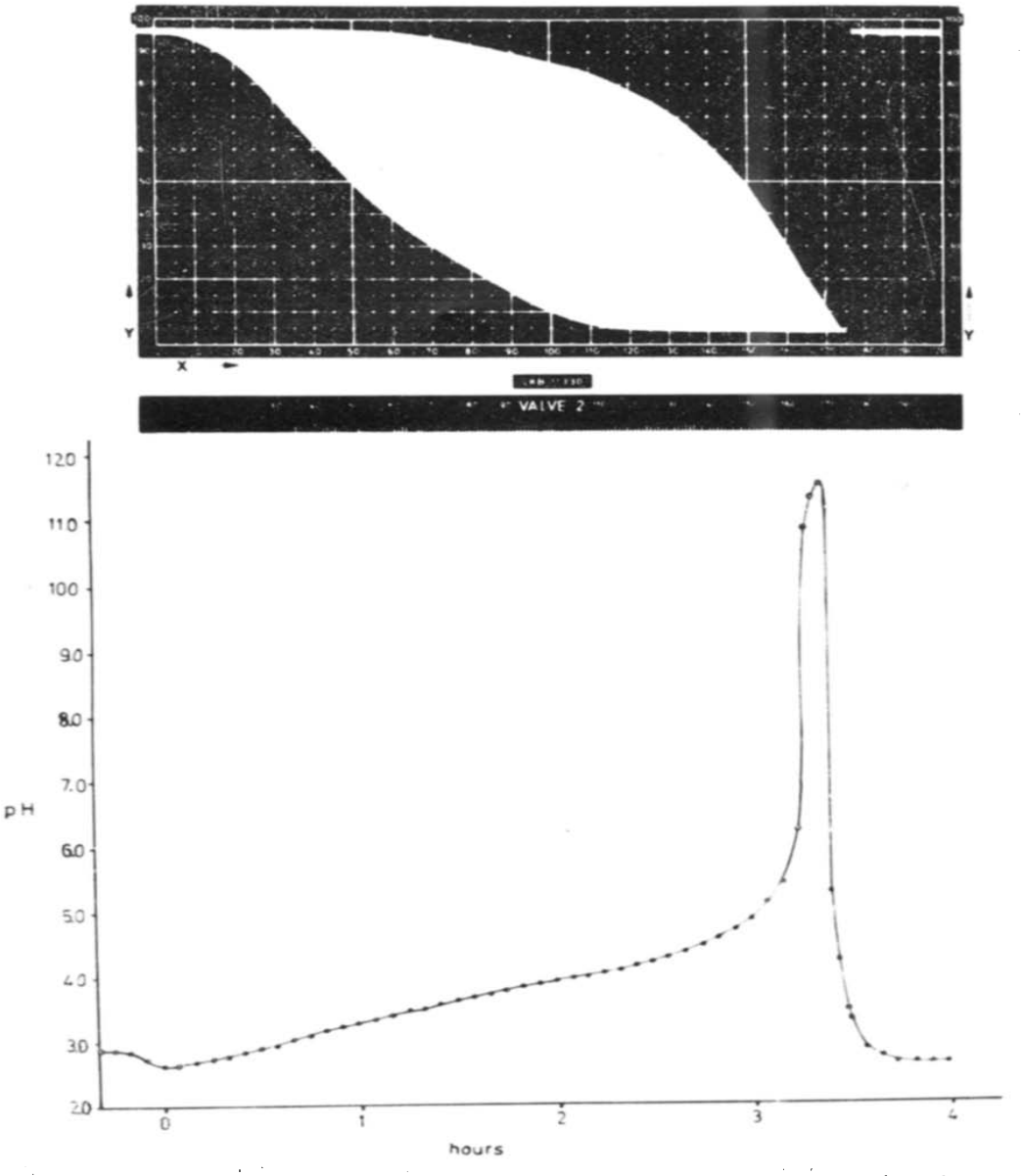

Fig. 6. The gradient curve chart scanned by the Ultrograd to give the pH gradient shown via mixing of the three buffers from reservoirs A, B and C (pH 2.58, 3.80 and 12.00, respectively). The pH before zero time (abscissa) is determined with the buffer of pH 2.72 in the buffer delivery lines from the mixer to the top of the columns (see text). The effluent pH obtained from the mixer at zero time is 2.63.

chart is cut into three fields according to the three buffers to be mixed (Fig. 6). The lower black field represents the buffer of pH 2.58, the white area in the middle the buffer of pH 3.80 and the upper black section corresponds to the buffer of pH 12.00 (1.8 *M* NaCl). The gradient curve chart was constructed using an empirical procedure and gives the same effluent pH curve that had been obtained previously from the Autograd[6]. The chart and the pH profile produced from it are given in Fig. 6. For optimal operation the buffer reservoirs should be adjusted to approximately equal heights as the mixer M and the two LKB valves V_1 and V_2 in order that the hydrostatic pressures on both sides of the valves are the same. Air bubbles within the buffer stream, set free by the intense mixing of the liquids in the mixer M, are removed by air traps, which are inserted in the buffer lines in the form of a lying T-piece. The first trap is located immediately after the Variport valve. A second trap is located immediately before the Milton Roy pumps so as to prevent air from entering the pumps, thus decreasing the volumetric input.

Taking advantage of the automatic gradient mixing device, the time-consuming procedure of filling five nine-chamber Autograds per day with exactly the same volumes of buffer became unnecessary. In addition, the use of a gradient mixer instead of discontinous buffer elution gives the advantage of an undisturbed baseline without buffer-jumps, which might cause difficulties in the automatic integration of the amino acid chromatograms obtained (Fig. 5). High accuracy in gradient reproducibility is another benefit given by the automatic gradient mixer. Hence the reproducibility of duplicate samples is ±2–3%. However, the resolution of the amino acids is not only a function of the gradient but also depends on the optimal adjustment of the length of the connecting buffer lines, the flow-rate through the column, the column temperature and the type of resin and its bed volume. The bed volume of the resin was carefully adjusted so as to give maximal resolution, which was obtained with *ca.* 9.5–10 ml of resin, which had been packed in small sections under the final working pressure throughout the packing procedure. A column temperature of 61° was found to give optimal resolution with the Chromobeads B resin. For regeneration of the column, pumping 0.2 *N* NaOH for 30 min followed by buffer for 15 sec was found to be adequate. A buffer of pH 2.91 was used for regeneration, as it was found that this pre-treatment followed by elution with a more acidic buffer from the gradient mixer increased the resolution of the first amino acids. Five columns prepared and treated in this manner have been in continuous use for more than 1 year in our laboratory without the need for repacking. Slight settling of the resin by about 3–5 cm occurred within this time, but this did not decrease the resolution.

CONCLUSION

It can be concluded that with a few changes to an existing amino acid analyzer, the apparatus can carry out single-column analyses in a 4-h cycle. The nine-chamber Autograd used for gradient mixing can easily be replaced with a commercially available gradient mixer with an appreciable gain in servicing time. The sensitivity of the entire system can be increased up to the 0.01-μmole level when columns of a lower than the usual cross-sectional area are used, as has been described. A control unit that can easily be constructed from available spare parts has been described

that facilitates automatic and continuous operation of the analyzer in carrying out five analyses daily. Taking advantage of newly developed resins for amino acid analysis, it is to be expected that increased flow-rates will enable an amino acid chromatogram of all protein-constituent residues to be completed within 3 h or less.

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