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DESIGN AND APPLICATIONS OF A MICROPROCESSOR-CONTROLLED SYSTEM TO OPTIMIZE PREPARATIVE LIQUID CHROMATOGRAPHY

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SUMMARY

A microprocessor-controlled instrument, able to pilot pumps, solenoid valves, fraction collectors as well as any other electrical appliances, was designed to improve the efficiency of our preparative liquid chromatography equipment. Facilities to select buffers, load samples, generate linear gradients and modulate the programmed elution processes by positive feed-back interaction with up to eight on-line detectors were included in the software. As many as 50 consecutive instructions may be programmed as simple coded mnemonics and executed on the 16 parallel output lines with respect to actual time and/or external feed-back signals. Sets of instructions may be stored on tape cartridges using a normal tape-deck. The main improvements brought about by use of this technology are greater reproducibility of elution patterns and improved resolution of the detected peaks. An application to the fractionation of protein from human serum is described and differences between methods are discussed.

INTRODUCTION

In most of the laboratories where proteins are purified, liquid chromatography (LC) is often used. During the last decade, major improvements in the design of LC equipment have resulted in a new generation of sophisticated columns, applicators, fraction collectors and detectors having increased efficiency. LC gels are now available as beaded, cross-linked matrices and derived products for ion-exchange or affinity chromatography[1–3]. The strengthened structure of these gels insures stable hydrodynamic properties and prevents large volume variations upon pH or ionic strength changes[4]. Thus, they may be regenerated within the column. This is man-

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datory for full automation, which may now be considered for the preparation of most proteins.

Using programmable flow selectors to perform the sequential steps of LC, automatic loading of samples, elution with several buffers as well as regeneration processes may be achieved[5,6]. However, some events cannot be programmed in advance, for example those actions taken consequent upon the results obtained, such as the modification of an elution process with respect to the current elution pattern. Such feed-back interactions can more easily be directed by a microprocessor since full-time survey of long lasting experiments by laboratory staff is uneconomic and tedious.

The aim of this work is to improve the management (automation of systems) and the overall efficiency of LC techniques (modulation of elution processes by feed-back). To this end we have designed and built BIDULE 1, the first prototype of a "Basic Instrument Directing Unattended Laboratory Equipment".

MATERIALS

LC equipment

The liquid chromatography columns were obtained from Pharmacia (Uppsala, Sweden). Cheminert connectors and valves from Laboratory Data Control (Riviera Beach, FL, U.S.A.), the Type 311 and 332 miniature electrovalves from Huba Control (Würenlos, Argovie, Switzerland), the UA-5 UV detector from ISCO (Lincoln, NE, U.S.A.) and the COM2e conductivity meter from Radiometer (Copenhagen, Denmark). The DE-52 cellulose was a product from Whatman (Maidstone, Great Britain), chemicals were reagent grade from E. Merck (Darmstadt, G.F.R.) and distilled, pyrogen-free water was supplied by the Hôpital Cantonal Universitaire. Human sera for routine analysis were collected in our laboratory, then pooled and stored at −25°C.

Electronic components

The MCS-85 System Design Kit and corresponding integrated circuits were purchased from Intel Corp. (Santa Clara, CA, U.S.A.), the DL-1416 four-digit, sixteen-segment alphanumeric displays from Litronix (Hitchin, Great Britain), the HCBB 75-W power supply from Power-One (Camarillo, CA, U.S.A.), the cases from Elma Electronic (Wetzikon, Zürich, Switzerland), the F-104 relays from Siemens-Albis (Dietikon, Zürich, Switzerland), and optocouplers and other electronic components in common use from local dealers.

METHODS

Chromatography of pooled normal human sera

DE-52 cellulose was recycled as described by the manufacturer and equilibrated in 20 m*M* Tris–HCl buffer, pH 8.2, degassed under vacuum. It was then poured into a column (70 × 1.6 cm) equipped with reservoir and sedimented at 32 ml/h overnight, to yield a 63-cm bed of packed anion exchanger. An adaptor was then fitted to the top of the bed and the flow-rate adjusted to 16 ml/h. A pool of sera was dialysed three times against ten volumes of 20 m*M* Tris–HCl buffer, pH 8.2, and

centrifuged for 1 h at 15,000 *g*. An aliquot (10 ml) of the dialysate was loaded on the top of the column. The column was washed with equilibration buffer to recover unadsorbed proteins, a linear gradient from 0 to 0.3 *M* NaCl in 20 m*M* Tris–HCl buffer, pH 8.2, was applied using either the standard two-vessels, siphon and magnetic stirrer method[7] or by the gradient generator of the process controller, as described below.

Design and construction of the process controller

Hardware. BIDULE 1 (Fig. 1) is a modular control unit for any combination of up to sixteen independent electrical appliances. It works like an array of programmable switches piloted by a quartz clock. The choice of the microprocessor was very limited since our development facilities included only an Intel 8080 based computer terminal and a cross-assembler for this family of processors. Basically, BIDULE 1 comprises an Intel development kit for the microprocessor 8085, as shown in Fig. 2. This kit was chosen since it includes almost all the elements needed: central processing unit (CPU); 2-kbytes erasable programmable read-only memory (EPROM) for the internal control program; 512-bytes random access memory (RAM) for user's instructions; parallel input/output ports for sixteen output and eight feed-back input lines and a timer to control the clock. In addition, it was one of the few kits which contain a 24-key keyboard and a display directly controlled by a dedicated circuit.

This choice allowed us to minimize hardware development. Minor modifications of the kit include the relocation of the six-digit, seven-segment display (clock) and keyboard upon the front and keyboard panels of the case, the addition of a twelve-digit, fourteen-segment alphanumeric display for the listings of the user's program and flags, as well as the implantation of optocouplers in the output and input

Fig. 1. BIDULE 1 prototype as currently used in our laboratory.

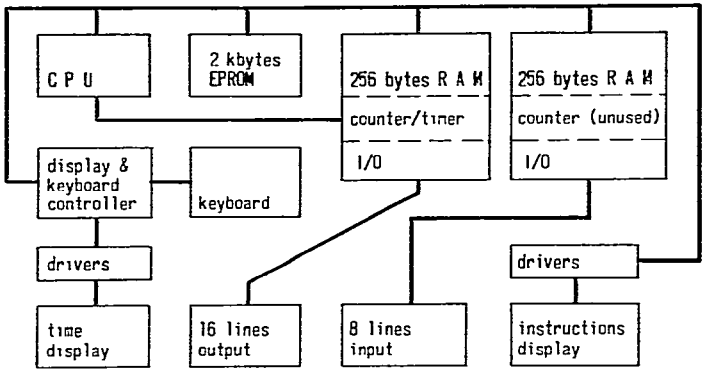

Fig. 2. Block diagram of hardware.

ports to protect the electronic components from external damage. The building and modifications of the kit were performed according to user's manuals[8,9]. A remote relay-box contains the power supply to satisfy the requirements of the electrical appliances to be used and is connected to BIDULE 1 through up to 25 m of multiple pole cable. The state of the input/output ports is continuously monitored by a row of light-emitting diodes (LED) on the front panel of both BIDULE 1 and the relay-box. Facilities offered by BIDULE 1 include on/off functions, generation of impulses and gradients as well as feed-back control of the processor by internal or external events.

Software. The internal software is written in ASSEMBLER[10], and is divided in two distinct parts.

(1) A kind of editor specially designed for BIDULE 1 which allows the user to give task-oriented instructions to be either immediately executed or stored in RAM memories for delayed execution. Short instructions control the state of BIDULE 1 and are used to start or stop the clock, to enable or disable the feed-back system and delayed execution and to read/write the user's program from/to a conventional tape-deck. The other instructions describe the state of the output ports with respect to time and/or changes in the state of the feed-back input lines. These instructions have the same twelve-character format, which may exceptionally be truncated on the right-hand side. The first two characters define the type of the instruction and are used to enter a set of instructions, to select feed-back registers, to modify immediately the state of the output ports or to clear all or part of the RAM memory. The next six characters are reserved to set the time at which the instruction has to be executed or to update the clock. These are followed by two characters used to define functions and their parameters which control the state of the output ports. These are usually on/off functions, generation of impulses or gradients. The last two characters indicate which of the sixteen output lines is concerned with the instruction. Extended error checks are provided to avoid the execution of illegal orders and the corresponding flags are displayed as descriptive words for convenience. Edit facilities such as "clear", "list" and "back-space" insure ease of correction, insertion or deletion of instructions within a program or of characters within an instruction.

(2) Internal software controlling the delayed execution of the instructions described above, with respect to the actual time displayed by the clock and the feed-back signals. Every time a new instruction is entered through the keyboard, BIDULE

I checks for immediate or delayed execution. In the latter case, the instruction is stored in the RAM memory. If the delayed execution mode is enabled, BIDULE I scans the stored instructions ten times a second, checks for conditions of execution and finally executes, or continues the execution of these instructions. The immediate execution of any instruction is always possible.

APPLICATIONS TO CHROMATOGRAPHY

The facilities offered by BIDULE I in the management of liquid chromatography systems may be placed in the following categories.

On/off selectors. The programmable on/off functions are used to start or stop pumps, detectors, chart recorders, fraction collectors as required, at any time within the range of the clock (99 h, 59 min, 59 sec) or according to external events. Two-way solenoid valves are also piloted to open or close different buffer reservoirs and to inject large samples, via T-shaped flow connectors. Three-way solenoid valves are used to divert flows from the main line, or to select one from two streams of solvents.

Impulses. The generation of single impulses from 0.1 to 25 sec allows one to pilot rotating valves and fraction collectors, and to inject very small samples without having to use sample loops and dedicated sample injection valves.

Feed-back. Up to eight parallel analogue feed-back input lines are used to connect external detectors such as a photometer, conductivity meter or pH meter. The signals are compared to a preselected threshold level (Fig. 3). If the signal potential crosses the threshold the programmed instruction is executed. This instruction

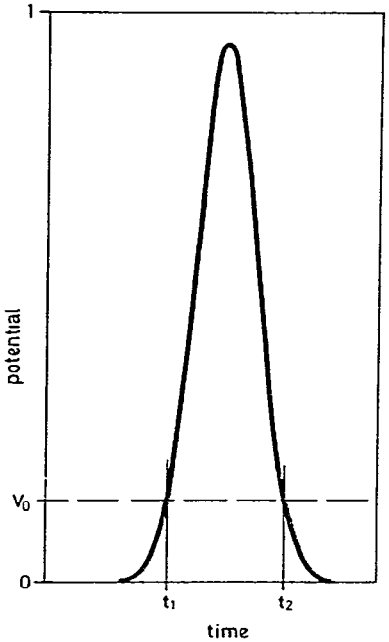

Fig. 3. Generation of feed-back signals. The threshold potential, V_0 (broken line), is chosen at a given level between 0 and 100% of the full scale. As the input signal (heavy solid line) crosses the threshold at times t_1 (activation) and t_2 (base return) the feed-back instructions programmed and stored in registers A and B are executed, respectively.

can be a jump of the clock to any given new time and/or any other standard instruction described above. Two independent feed-back signals are recognized on each of the input ports: these are "activation" (increasing potential across the threshold) or "base return" (decreasing potential across the threshold) signals which respectively detect the beginning and end of peaks during chromatography. In addition, an internal software loop simulates external feed-back signals. This allows recycling of programs or jumps of the clock to avoid execution of a segment of program.

Gradients. Linear gradients are generated by pumping increasing amounts, y, of solvent Y in proportionally decreasing amounts, x, of solvent X, step by step as shown in Fig. 4. The electric pulses applied to the solenoid valves generate a stair-like gradient resulting in a continuous linear inclined plane of the same slope beyond an appropriate mixing chamber. This stair climbs from the initial level, N_0, to the final level, N_n, through $n - 1$ intermediate levels, N_i (n-step staircase, $0 \leqslant i \leqslant n$, Fig. 4A). The length, L, of each step is a multiple of the length, C, of the programmed repetitive cycle generating the impulses (Fig. 4B)

$$L = mC \tag{1}$$

where m is a positive integer ($1 \leqslant m \leqslant 250$) chosen from a table of 30 selected values. The length, C, of a cycle depends on the number, n, of steps chosen, and on the length of the base time, z:

$$C = nz \tag{2}$$

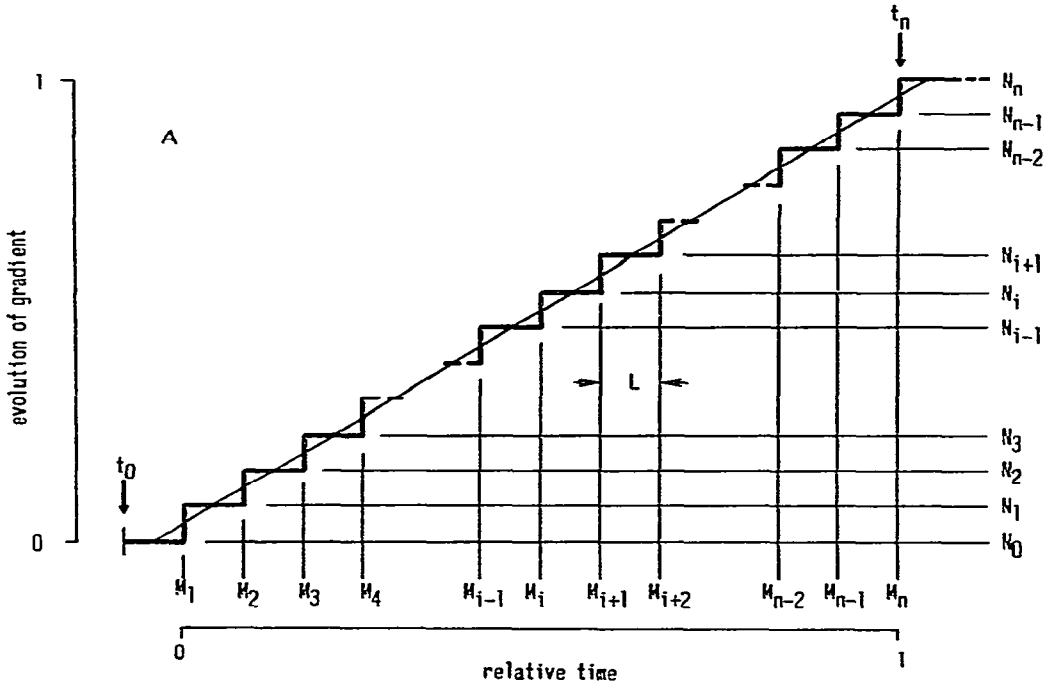

Fig. 4.

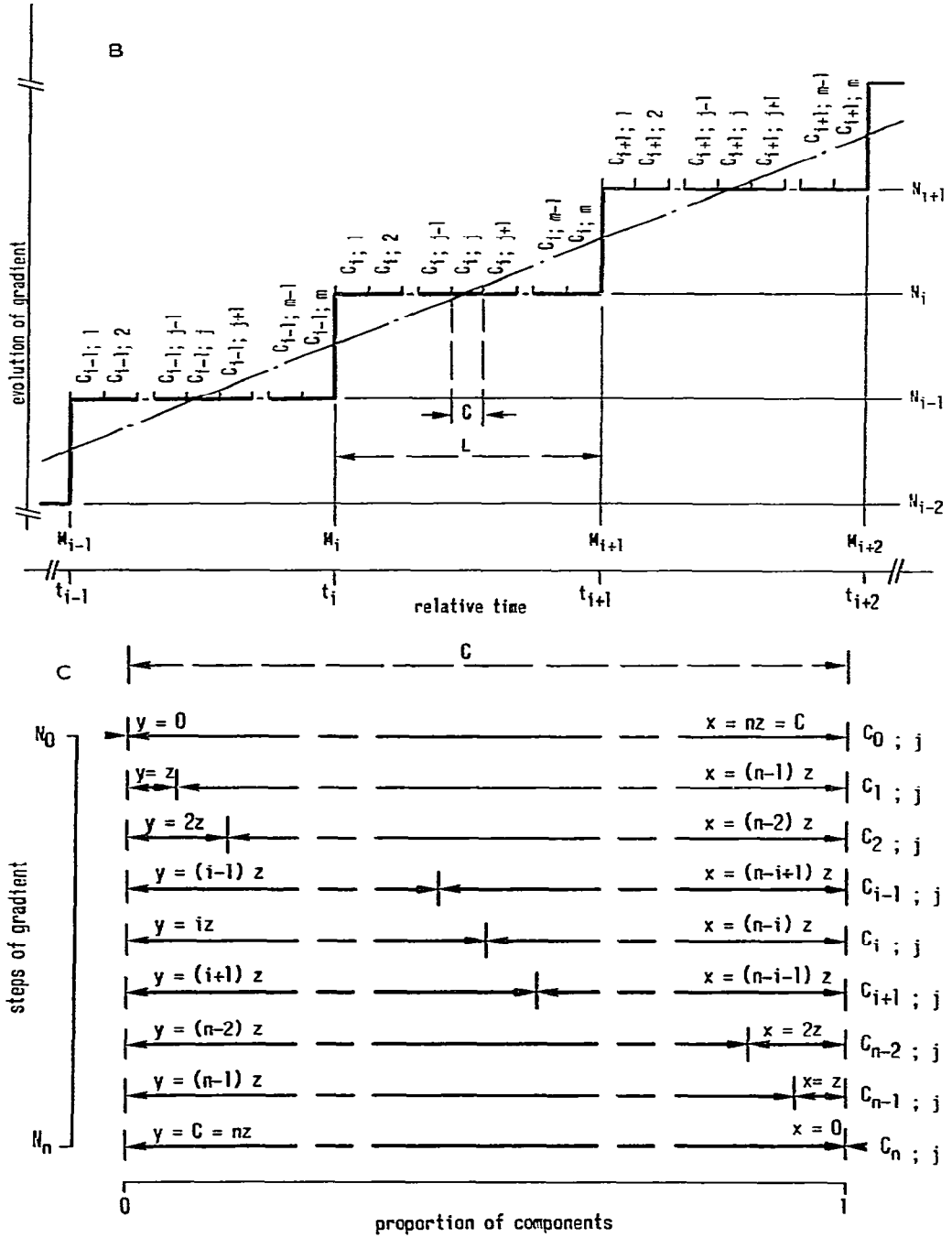

Fig. 4. Principle of generation of linear gradients. A, Stair-like gradient showing steps, M_i, of length L, levels N_i and duration from initiation (t_0) to achievement (t_n), as well as the mean slope obtained beyond the mixing chamber. B, Modulation of the slope through repetition of cycles C_i. n = Number of steps; m = number of cycles C_i per step; $C_{i;j}$ = single cycles of impulses, $0 \leqslant i \leqslant n$, $0 \leqslant j \leqslant m$; C = length of a single cycle $C_{i;j}$. C, Generation of sequential cycles by the processor. z = Smallest increment of time (base time = 0.1 sec, see text); i = number of the cycle within the sequence, $0 \leqslant i \leqslant n$; x_i, y_i = proportions of solutions X and Y, respectively, delivered within cycle $C_{i;j}$

Since the response time of electromechanical components piloted by BIDULE 1 to generate gradients (electrical relays, solenoid valves) is the limiting factor of the system, the base time was fixed to the lowest possible value allowing reproducibility, *i.e.*, $z = 0.1$ sec. This value determines the smallest useful increment (Fig. 4C). The composition of each cycle with respect to both of the gradient constituents X and Y is given at any time, t_i, by the relations

$$C = x_i + y_i \tag{3}$$

$$y_i = iz \tag{4}$$

$$x_i = (n - i)\, z \tag{5}$$

where x_i and y_i are the respective amounts injected during the cycle C_i as illustrated in Fig. 4C. The conditions for the initial and final levels are then given by $y_0 = 0$ and $x_n = 0$ respectively. From Fig. 4A, we find that the duration of the gradient is determined by:

$$t_n - t_0 = nL \tag{6}$$

Substituting the L value from eqn. 1 and the C value from eqn. 2 into eqn. 6 we obtain the duration of the gradient with respect to the directly programmable parameters only:

$$t_n - t_0 = mn^2 z \tag{7}$$

Both parameters m and n can be selected through the keyboard. The number of steps, n, is chosen before the gradient starts and cannot be changed during gradient generation ($n = 25k$, $1 \leqslant k \leqslant 10$). The repetition factor, m, of each cycle is selected as a part of the instruction setting the gradient, and can be modified at any time. This factor allows the modulation of the slope of the gradient, either by standard instruction or by feed-back control. The shortest gradient possible lasts 62.5 sec ($n = 25$, $m = 1$), the longest lasts 18 days, 2 h, 1 min and 40 sec ($n = m = 250$)! Non-linear concave or convex as well as other special gradients may be built from multiple consecutive modifications of the slope and/or juxtaposition of several consecutive gradients. Arbitrarily, the value $m = 0$ indicates an instruction which suspends the evolution of the gradient (slope = 0), until another value is selected. Such an instruction allows one to modulate the development of a gradient with respect to the actual elution pattern, using the feed-back signals from an on-line detector connected to BIDULE 1, as shown in Fig. 5.

EXAMPLES OF APPLICATION AND DISCUSSION

As an example, the fractionation of proteins from pooled human sera on DEAE-cellulose is discussed for two types of elution processes: a conventional two-vessel, syphon and magnetic stirrer gradient mixing chamber (Fig. 6A), and the gradient generated by BIDULE 1 and modulated by the UV detector through the

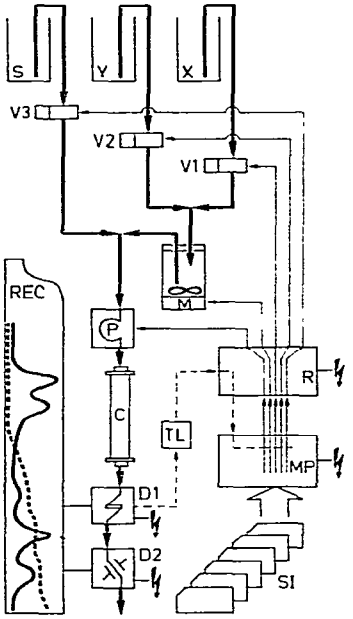

Fig. 5. Schematic diagram of the equipment used for the generation of the gradients by the microprocessor, with modulation by feed-back signals from a UV detector. S = Sample vessel; X, Y = buffer reservoirs for first and second gradient components, respectively; V1, V2 = solenoid valves; V3 = sample injection valve; M = mixing chamber (1 ml) with magnetic stirrer and bubble trap; P = pump; C = column; D1 = detector 1 (UV monitor); D2 = detector 2 (conductivity meter); REC = recorder coupled to detectors; R = relays; MP = microprocessor (BIDULE 1); TL = threshold level adjustment; SI = set of instructions (program) to the microprocessor.

feed-back facilities (Fig. 6B). Using a peak separator able to detect the minima of the absorbance curve ($\delta A/\delta t = 0$), the number of fractions collected increases from 7 to 13. Their degree of separation can be adjusted to individual requirements by selecting an optimum threshold potential for the feed-back system. Generally, this optimum value should be estimated from the type of column to be eluted (size, ratio of length to section, type of packing, flow-rate) and from the complexity of the mixture to be purified and results expected (number of components in the sample, number of peaks to separate, degree of similarity between single components to be eluted in discrete fractions, behaviour of single components with respect to the column packing). Too high a threshold will decrease the resolution, since the gradient will be incremented before the elution of the preceding peak is completed. Lowering the threshold produces some longer intermediate levels, an elution at a lower ionic strength and, thus, a concomitant spreading and dilution of the fractions. This is best understood by considering Fig. 6B, where most of the well separated, sharp peaks are eluted just after an increase of the ionic strength, in contrast to the shoulders and broader peaks which are eluted at the end of the intermediate levels of the gradient. For a given column at a constant flow-rate, the shift between the programmed gradient injected on the column (Fig. 6B, solid line) and the resulting gradient flowing out through the detector (Fig. 6B, dotted line) is a direct measure of the liquid phase volume, V_1,

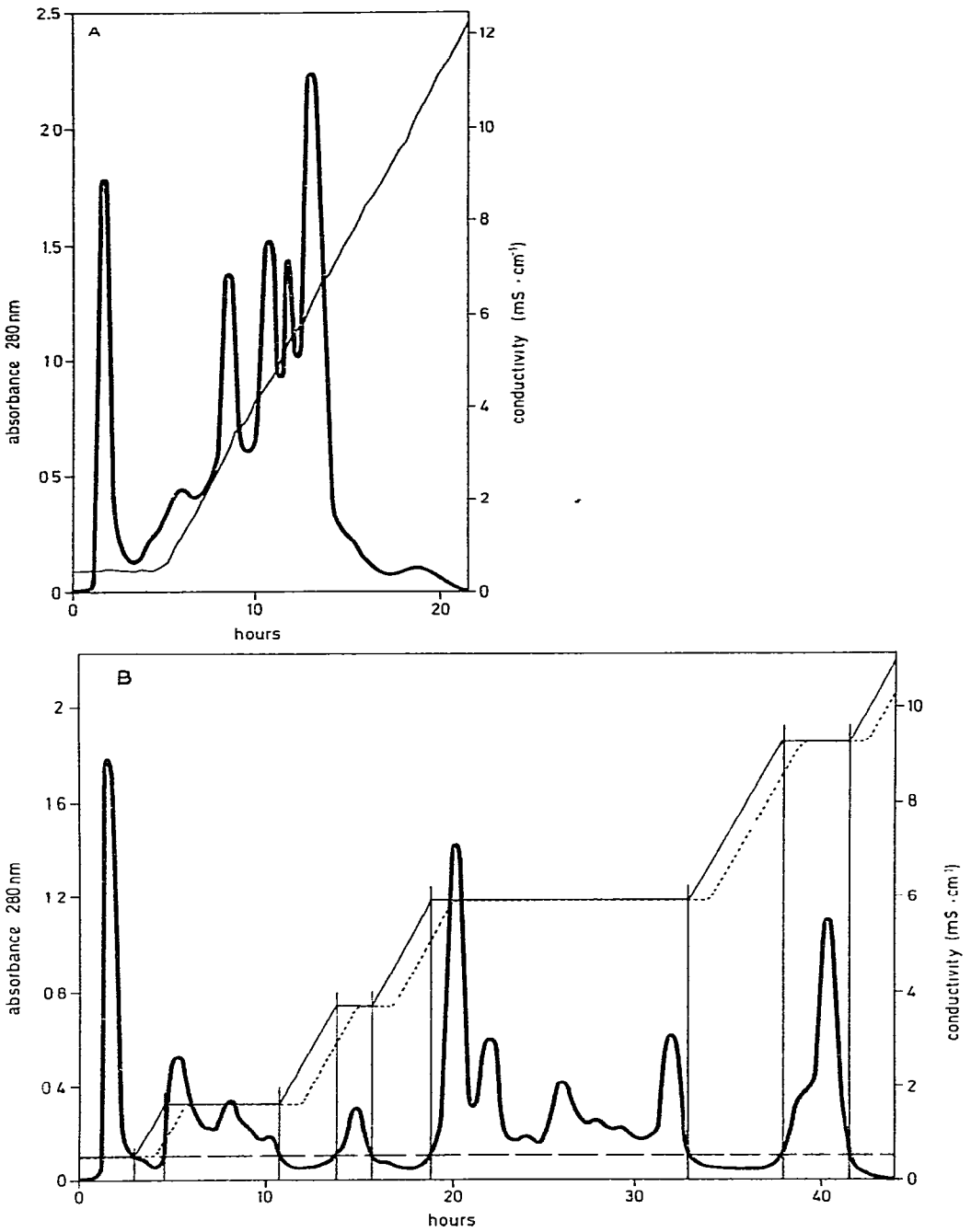

Fig. 6. Chromatography of proteins from pooled human sera on DE-52 cellulose. Column: 63 × 1.6 cm. Flow-rate: 16 ml/h. Buffer: 20 m*M* Tris–HCl, pH 8.2. Gradient: 0 to 0.3 *M* NaCl in buffer. A, Manual operation. Sample loaded through the pump, gradient formation by two-vessel, siphon and magnetic stirrer technique. B, Microprocessor-controlled operation. Sample loaded by sample injection valve, gradient generated by microprocessor and modulated as shown by feed-back signals from the UV detector. Solid line: generated gradient as injected onto the column. Dotted line: gradient as measured in the exit liquid stream. Broken line: threshold level. Heavy solid curve: absorbance at 280 nm.

within the column as a function of time. Thus, the optimum length, L, for each step of the gradient should be $L = V_1$. However, such a value for L may yield poor results if the column is large and/or the flow-rate is low, since both these parameters will contribute to flattening the slope of the gradient.

Among the facilities offered by BIDULE 1, the feed-back system provides many opportunities of dealing with automation problems and with events which are expected but which cannot be scheduled. Beside modulation of the eluting gradient discussed above, other possible uses of the feed-back system are briefly presented here to illustrate the broad range of potential applications in chromatography. When connected to temperature and pressure detectors, buffer level gauges or any other sensors installed within the chromatography set-up, the microprocessor may be programmed to modify these conditions, switch off some defective apparatus, stop the experiment and/or trigger alarm devices (crash programs). When connected to an on-line peak detector and piloting a three-way solenoid valve, BIDULE 1 is a perfect tool for the direction of recycling chromatography[11]: bleeding and recycling sequences may automatically be alternated with respect to the actual position and degree of separation of peaks (dynamic evolution of programmed events). With an additional pulse-piloted rotating valve to divert the flow stream from the main line, BIDULE 1 may be used instead of a fraction collector for several discrete fractions during repetitive chromatography with multiple injections of sample.

CONCLUSIONS

Over a year of continuous tests, we have demonstrated the ability of BIDULE 1 to successfully control the operation of up to three peristaltic pumps and twelve solenoid valves delivering samples and buffers to three distinct parallel flow streams. The main improvements achieved in our laboratory by this simple process controller are simplified management of LC equipment, better reproducibility of results and higher resolution of ion-exchange chromatography. Each of our LC columns may now be used 24 h a day, since loading of samples, changing buffers or eluting and recycling processes require no human intervention. This saves time and valuable reagents, and produces more results, both quantitatively and qualitatively. Furthermore, the reproducibility is improved since, once given the parameters of chromatography, the processor excludes even minor changes in the conditions of experimentation. Finally, coupling of a UV detector to the processor (feed-back) in order to modulate the slopes of gradients according to the development of the elution considerably increases the resolution of a given separation, as demonstrated in Fig. 6.

Some applications of BIDULE 1 to specific purification procedures of proteins will be described in separate papers. We are currently testing an automatic multi-column LC system designed to yield weekly highly purified single protein fractions from human plasma.

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