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Analytical Techniques for Cell Fractions

XIX. The Cyclum: An Automatic System for Cyclic Chromatography

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An automatic system, termed a Cyclum, is described which allows column chromatographic separations to be repeated precisely a large number of times. Provision is made for the adjustment during operation of parameters such as equilibration, wash, elution, and sample flow times and duration of fraction collection. The system is applicable to both analytical and preparative use in various types of column chromatography (e.g., affinity, gel filtration, ion-exchange), but has been especially developed for separations based on immunosorption.

The isolation of trace constituents, such as human cancer-associated antigens, from complex mixtures requires the use of sensitive separation techniques which may be used either analytically or on a preparative scale (1). Of the available methods, so-called affinity chromatography using immobilized antibodies, antigens, substrates, or other stereospecific substances holds the greatest promise (2). However, to use these methods one generally requires either samples of the antigen to be isolated so that antibodies against it may be prepared, or detailed knowledge of binding sites on the molecule to be isolated so that suitable

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nonimmunological adsorbents may be made. In attempts to search for tumor-associated macromolecules which may be diagnostically useful, it is necessary to develop very general methods which allow differences (preferably qualitative ones) between samples of urine, plasma, or cell extracts from normal subjects and from cancer patients to be detected, and the substances responsible for the differences isolated. Once this is done, and the molecules of interest characterized, preparative methods specific for those molecules may be developed. Thus there are two problems—the initial discovery and characterization, and secondly routine isolation. The rationale behind our tumor antigen search studies has been described in detail elsewhere (3–9), and preliminary reports of the isolation methods to be described have appeared (10,11).

A fundamental notion is to employ only methods which are both preparative and analytical and which may be scaled up easily by merely repeating them automatically using an automatic cycling (Cyclum) system. To do this rapidly and efficiently requires that the methods employed involve no slow, time-consuming steps such as the equilibration of ion-exchange columns where large changes in ionic composition and pH are required. We have therefore avoided ion-exchange columns initially and have used instead affinity chromatography and gel filtration. Columns of either immobilized antigen(s) or antibodies have been made, and have been used either for subtraction of *contaminant* antigens or antibodies, or for isolation of *desired* antigens or antibodies (1). A sequence of steps termed "bootstrapping" has been developed which may be illustrated by the problem of isolating pregnancy-associated antigens from human pregnancy serum. The first step is to immunize goats or rabbits with normal (nonpregnancy) serum. The antisera obtained are then passed through a column on which normal serum proteins have been immobilized. Antibodies against normal serum proteins are thereby removed from the antiserum, eluted, dialyzed free of eluting solution, and recovered. These antibodies are immobilized to make antinormal serum proteins column (or contaminant-antigen subtraction column) which is in turn used to remove normal serum proteins from pregnancy serum. The small amounts of pregnancy antigens which fall through such a column are then used to produce antisera, and the IgG from such sera used to produce a so-called "first generation" column for isolating pregnancy antigens directly from pregnancy serum (desired-antigen subtraction). By repeated use of such a column sufficient pregnancy antigen(s) may be obtained to prepared a column of immobilized pregnancy antigens. The latter column in turn may be used to prepare IgG specific to pregnancy antigens in large quantity for the preparation of a specific IgG (or second generation) column for more efficient desired-antigen subtraction. With the latter, and with fairly rapid cyclic use, much larger quantities of antigen may be prepared and used for hyperimmunization, and

also for characterization, especially for determination of molecular weight. If the antigens present in the mixture differ markedly in molecular weight, then the final-product antigen mixture may be fractionated on that basis into individual antigens which are then used to prepare monospecific antibodies, monospecific antigen or antibody columns, and finally, quantities of pure antigen.

For all this to work, two basic requirements must be fulfilled. First the necessary apparatus for efficient cyclic column utilization must be constructed, and secondly, methods must be devised which allow affinity columns to be used many times over without degradation of either the immobilized or the adsorbed and eluted antigens or antibodies.

The development of instrumentation is described in this paper, and the evolution of specific separations methods is described in the next (12) and subsequent ones. Note that if each step in a three-stage series has an amplification factor of 100 (for example, contaminant subtraction, immunization, and desired-antigen subtraction), an overall amplification of 10^6 could theoretically be obtained. An analytical system in which a series of different samples is applied sequentially to a column has been previously developed by one of us (13). Methods for rechromatographing samples through the same or a series of columns have also been described (14–16), and a variety of timing, valving, and switching devices for programming chromatographic columns, and for collecting fractions either from identical runs or from specific peaks are available from commercial sources. However, none of these combine the requisite elements in a way which solves the problems described here.

It is important to distinguish four types of automatic chromatographic systems, which are: (a) analytical systems for analyzing a series of different samples by the same method, (b) preparative systems in which one peak is recycled repeatedly through one column to purify it, (c) preparative systems in which fractions from one separation are rechromatographed on others automatically to achieve a number of separations, and (d) preparative systems in which the scale of the separation is controlled by the number of repeated identical preparative cycles. For convenience we have adopted the term "Cyclum" for the latter system.

THE CYCLUM SYSTEMS

Eight versions of the complete system, illustrated diagrammatically in Fig. 1, have thus far been built. Two slightly different versions of the system (A and B) are shown in Fig. 2. The requirements are for a system in which the following may be done cyclically in a flexible manner:

1. A sample of known volume applied.
2. Buffer solution run through to wash out unbound antigens or antibodies.

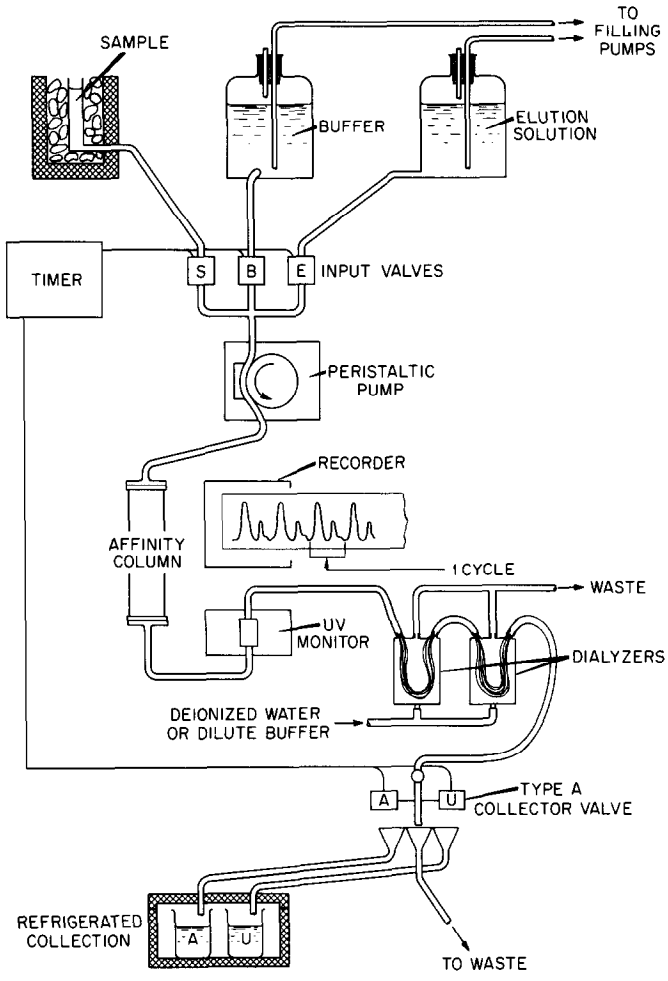

FIG. 1. Schematic diagram of Cyclum with type A collection valve. Input valves are programmed to feed buffer, sample, buffer, and eluting solution in that order cyclically through an affinity column. Separations are recorded using ultraviolet absorbance, and unbound (*U*) fractions and adsorbed (*A*) fractions collected after dialysis using a three-way type A collector valve.

3. The peak of unbound material collected.
4. An eluting solution run through the column.
5. Effluent dialyzed to remove the eluting solution.
6. The peak of eluted material collected.
7. Buffer pumped through the column to wash out the eluting solution.
8. Sample applied to restart the cycle.

Refrigeration of the sample and collected fractions is provided for, and the timing system is designed so that at the end of a preset number of

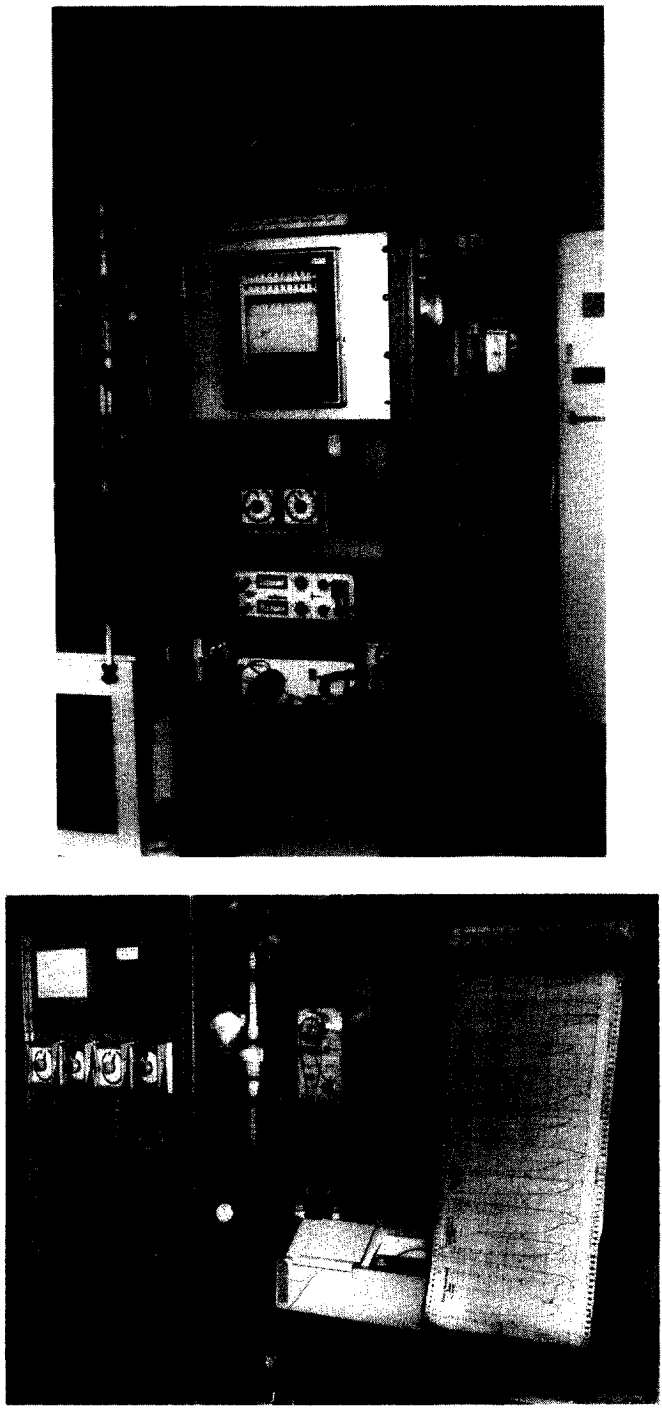

FIG. 2. Two versions of the Cyclum system. (A): System in upper photograph with fixed wavelength, linear with absorbance recording, and type A timer and three-way collection valve. (B): System in lower picture with spectrophotometer monitor, linear with percent transmittance recording, and type B timer and eight-way collection valve.

cycles the column is extensively washed and the entire system is shut down.

Several timing systems have been devised, and two (types A and B) are described here. The basic requirement is that the lengths of *all* intervals be separately adjustable. Thus the length of time the sample line is open (which together with pump flow rate controls the sample volume), the buffer and eluting solution flow times, and the time of initiation and duration of sample collection should be easily altered as required for the actual separations being made. These adjustments are made during the first few cycles, and fine tuning is done as required during the course of subsequent cycles.

All fluid lines are arranged so that accidental leakage, if it occurs, does not leak into electrical components.

Reservoirs. Three (and in one experimental version four) reservoirs are provided to supply liquid to the column. To insure that a slight positive pressure exists through all parts of the system, these are mounted on the top of the cabinet in a stainless steel pan which has a drain hole to waste. Wash and eluting buffer reservoirs are of 8-liter capacity, and all reservoirs in one room are connected through Tygon lines to large (40-liter) reservoirs at bench level. Peristaltic pumps with timers are used to fill individual reservoirs on the Cyclums, the timers serving to insure that these are not overfilled. The sample reservoir is usually of relatively small volume and is either placed in a small plastic foam box full of crushed ice, or may be placed in a small refrigerator at the bottom of the cabinet. In the latter case there is some likelihood that air bubbles will occur in the line.

Inlet selector valves. After much experience with a variety of solenoid valves, a very simple valving system was devised with spring pressure valves[6] pinching silicone-rubber tubing, and the spring closure being opposed and the valve opened by a solenoid (Fig. 3). Thus all valves are normally closed, and the energizing circuits are designed to allow only one valve to be opened at a time. The exit lines from the valves are brought together through a small four-way connector,[7] and the single emerging line flows to the peristaltic pump.

Pump. Several different pumps have been used, the most convenient ones being those allowing continuously variable control of pump speed.[8]

[6] Model AVSC-115, Clippard Instrument Laboratories, Cincinnati, Ohio. This is a solenoid actuator in an aluminum housing. We bore a hole through the back of the housing, and introduce therein silicone rubber tubing, so that the solenoid plunger, which normally actuates some outside device, presses in its rest position on the tubing, shutting off flow of liquid through it.

[7] Small Parts, TC-18/4, 6901 N.E. Third Ave, Miami, FL.

[8] We have used mostly the Buchler 4-channel Polystaltic pump, Buchler Instruments, Fort Lee, NJ.

FIG. 3. Three way solenoid-operated inlet valve. The valves pinch silicone rubber tubing and are normally closed. Activation of the solenoid opens the valve. To insure that two valves are never open at the same time, but that one is always open when the pump is operating, current to the solenoid switches is turned on with the pump. Two DPST switches control the solenoids. The first energizes either the sample valve *or* the second switch. The second switch activates either the buffer valve or the eluting solution valve. The three lines from the valves are brought together through a four-way connector and fed into the pump.

Columns. One-inch columns with packed bed lengths ranging from 15–40 cm have been generally employed, although columns of other dimensions may also be used. Note that the same timing applies to columns of different diameter but of the same length if the same flow rate per unit of cross-sectional area is used. A variety of columns are available, however in the present work standard 1 in. Pyrex pipe has been used together with commercially available adapters.[9]

Monitors. A number of different monitors have been used including those using fixed-wavelength detection,[10] and two based on spectrophotometers[11] which allow choice of one or several wavelengths. The important choices are those of most useful path length, and between recording linear with absorbance vs linear with percent transmission. While individual preferences may differ, for general use we have found absorbance at 280 nm with a 0.2 cm flow cell,[12] and recording linear with percent transmission to be most useful. No range changes are required, small peaks are expanded at the low end so that they are sensitively detected, and information on absorbancy changes in very dense peaks is still retained, though in a very compressed form, and no information is

[9] Pharmacia Fine Chemicals, Piscataway, NJ.

[10] UV Monitor, Model 1286, Laboratory Data Control, Inc., Riviera Beach, FL.

[11] Beckman DB and Beckman DU spectrophotometers, Beckman Instruments, Spinco Division, Pal Alto, CA.

[12] The Laboratory Data Control flow cell that came with the instrument was used; for the spectrophotometers, we constructed our own upward-flow cells.

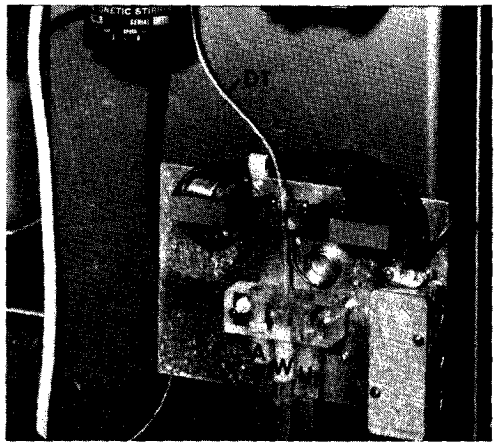

FIG. 4. Three-way (type A) collector valve. In the center (rest) position, the drip tube (DT) is over the collection funnel leading to waste. The tip is moved to the right to collect *U* (unadsorbed) protein and to the left to collect *A* (adsorbed fraction). *U* and *A* collection lines lead to refrigerated collection storage, while *W* leads to waste.

lost because of failure to change ranges. The recorders[13] used should have at least one event marker to indicate when fractions are actually collected. For developmental work, marking of when the sample is applied, and when buffer changes occur, is also useful.

Dialyzers. Two fiber dialyzers[14] are used in series and are fed with deionized water which may be turned off automatically when the "*U*" (unbound or unadsorbed) sample fraction is coming off, resulting in retention of sufficient buffer salts to keep globulins in solution. With most eluting solutions, enough salts are left in the stream to prevent globulin precipitation. Should this not be the case, or should a pH change be desired, a stream of concentrated buffer may be pumped into the stream of deionized water before it enters the second dialyzer. When only one stream of water is fed into the dialyzers, it should enter the bottom of the second dialyzer first, and from there to the bottom of the first (closest to the column) dialyzer.

Collector valves. Valving on the collection end is done in such a manner that no back pressure can be developed, i.e., the valve is always open to atmospheric pressure. Two types of valves have been employed, the first (type A) is simply a three-way collector valve consisting of a drip tube positioned over a strip of three funnels (Fig. 4). The rest position is with the drip tip over the center funnel which leads to waste. By

[13] Honeywell Electronik 17, or similar. Minneapolis-Honeywell Regulator Co., Brown Instruments Division, Philadelphia, PA.

[14] Bio-Fiber 50 Beaker Dialyzer, Bio-Rad Laboratories, Richmond, CA.

energizing either of the two side solenoids,[15] the drip tip may be moved to either side to collect either the unbound or the adsorbed peaks. This type of collector valve is useful for affinity chromatography where only two peaks are to be collected.

For gel filtration separations, or where antibodies of different affinities are desired, more fractions need be collected, and a more complex collection valve is needed. If conventional fraction collectors are used for this purpose, many fractions are generally collected and then pooled, after reference to either the absorbance monitor chart or to other analytical data, to yield a few combined samples. The precision with which this may be done increases with the number of fractions collected. If only small numbers are collected, part of the resolution may be lost, since one fraction may contain material from two peaks. If the collection valving system can be properly programmed, then pooling can, in effect, be done *during* collection, and only the stream segments actually desired retained. For these reasons a rotary collection valve (type B) was developed which allows fluid to drip into any one of eight collector funnels, one of which is led to waste. The dwell time over any one collector funnel may be separately controlled, and if desired the collector will return to waste between any, all, or no collection points. In this way only the samples ultimately required are collected.

The type B rotary valve is shown in Fig. 5 and consists of an upper rotatable plate to which the input line is flexibly attached so that it does not twist when the upper plate turns, and a lower plate incorporating eight funnels. The upper plate is driven by a small electric motor through a beaded-chain drive in response to signals from the timer. By each funnel is a small microswitch embeded in a hole, with a steel ball above it. The upper plate depresses all the balls except one, thus closing all the switches except the one poisitioned by the drain line, which is the one being actuated. This is done by providing one small curved recess in the upper plate. When another switch is energized, the upper plate revolves until the energized switch is reached and opened. The timer circuit is designed to always energize either the switch by the waste funnel, or *one* other switch. This system allows samples to be collected in any order, and for periods of drain to waste to be interspersed between collections, and, using the type B timer, for all collection of drain-to-waste periods to be varied and adjusted separately. In addition the timer may be adjusted to collect different numbers of samples, and each cycle may include one to seven fractions in addition to waste.

Collection and refrigeration. Samples are drained through separate

[15] The drip-tip carrier connects the two solenoids. A coil spring keeps the drip tip over the waste portion, until a solenoid pulls it to one side or the other.

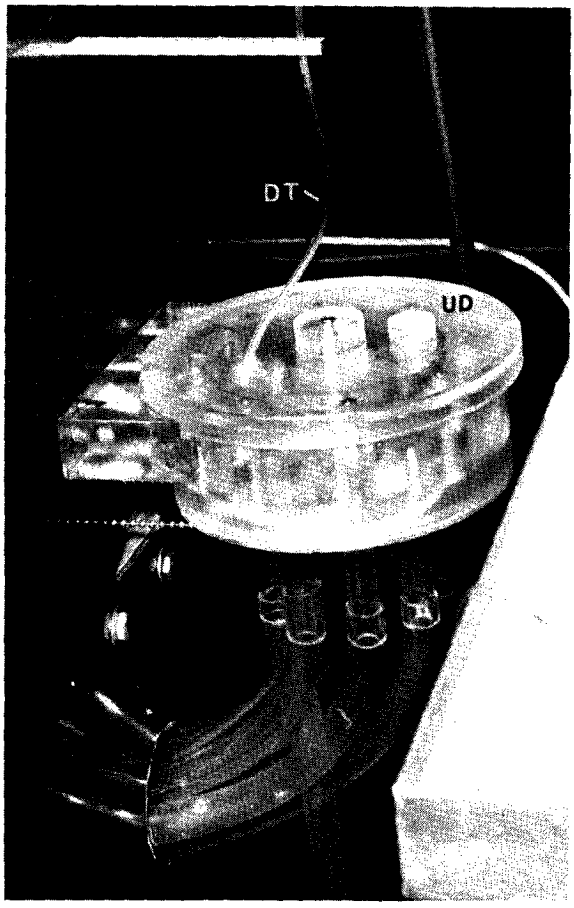

FIG. 5. Eight-place type B rotary collection valve. The drive motor (not shown) is actuated by the timer through any one of eight microswitches located next to the collection funnels in the base. The motor drives the upper disk (UD) with attached drip tube (DT) until it is next to the switch being energized. A detent arrangement in the upper plate opens the switch when the upper plate is correctly located. Seven fractions may be collected, one funnel leading to waste.

collection tubes into a small portable electric refrigerator[16] mounted in the bottom of the cabinet (Fig. 6), and into a rack containing seven calibrated plastic collection tubes. The refrigerator cover is arranged to open when the sliding shelf is pulled forward. When only two peaks are collected, the collection tube rack is removed, and two large beakers used instead.

Timing. The sequence of events occurring in cyclic affinity chromatography is illustrated in Fig. 7 for the A system, and both A and B

[16] Bernz-o-Matic, Model TX-2000V or equivalent. Bernz-o-Matic, Rochester, NY.

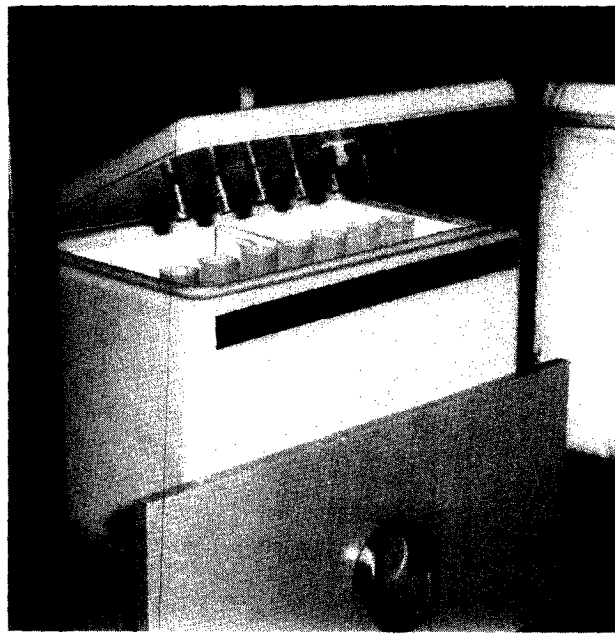

FIG. 6. Refrigerated collected-fraction storage.

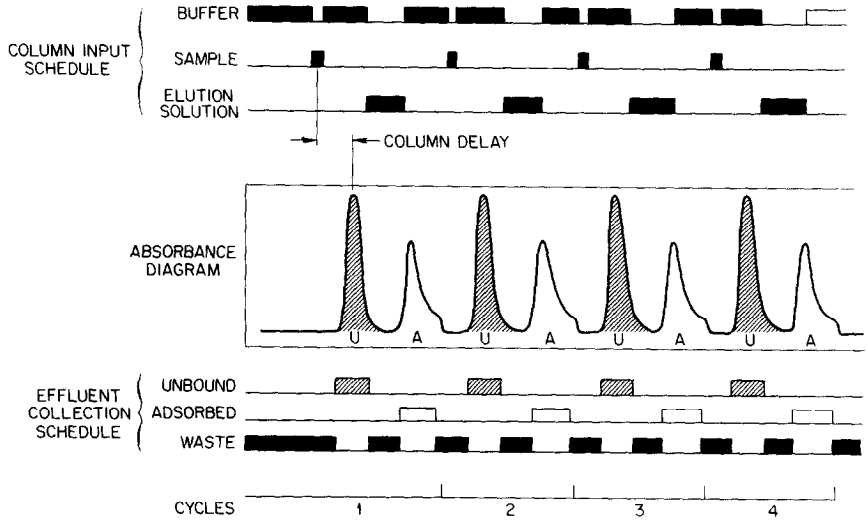

FIG. 7. Diagrammatic representation of Cyclum timing requirements for affinity chromatography. Above: Schedule for solution flow into the column. Buffer, sample, buffer, and elution solution repeat in that sequence. Center: Absorbance diagram showing alternate appearance of unbound (*U*) and adsorbed and eluted (*A*) peaks. Below: Collection schedule of *U* and *A* peaks. The column delay time depends on the column volume and flow rate.

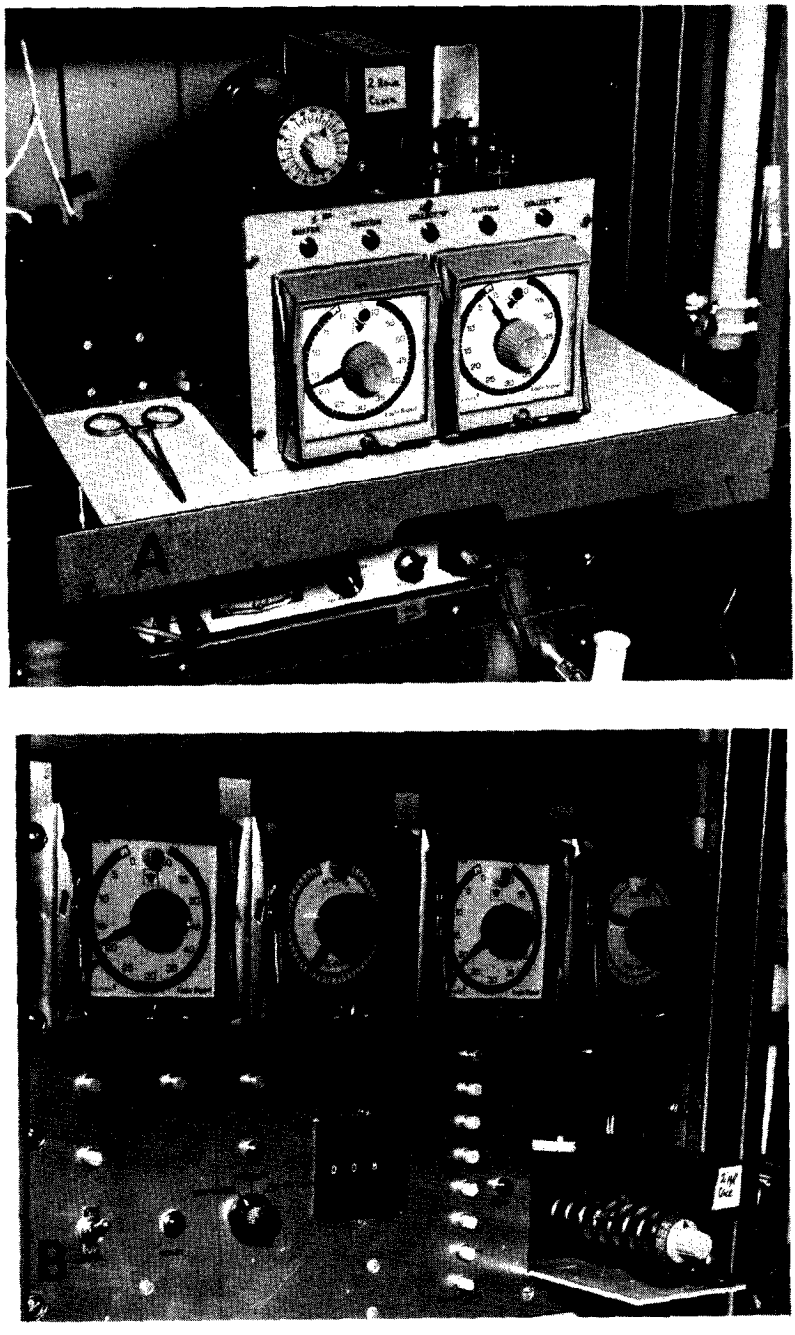

FIG. 8. Cyclum timers. Above: type A; Below: type B. Both control input of buffer, sample, and eluting solutions on similar schedules. Type A operates a three-way collection valve, type B actuates an eight-way valve.

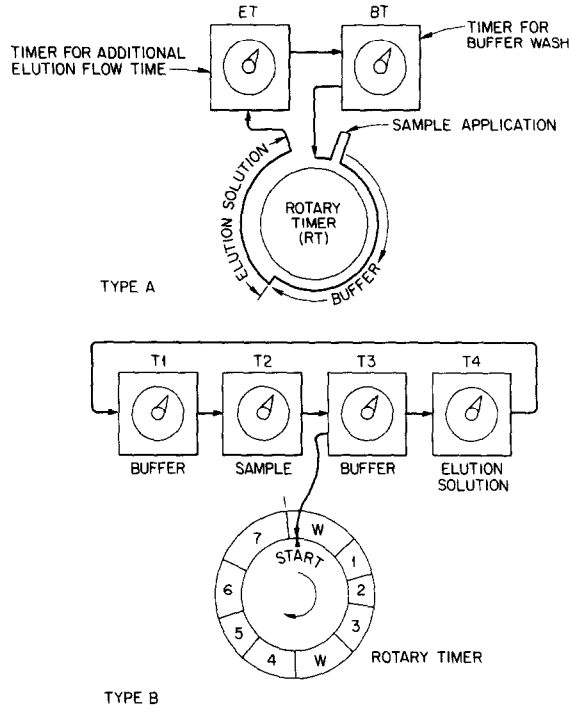

FIG. 9. Arrangement of Cyclum timers. Type A timer (above) in which three timers activate each other in circular sequence. Initially timer BT times a column wash with buffer at the end of which the rotary timer RT is started. This timer controls samples addition, buffer flow to allow unbound (*U*) proteins to pass through the column, and the introduction of eluting solution into the column. (Control of sample collection is also done by the rotary timers as described in the text but is not shown here.) The type B timer (below) employs a ring of timers (T1, T2, T3, and T4) to control buffer, sample, and eluting solution flow as indicated, while fraction collection is controlled by a separte rotary timer activated when T3 is activated. The sequence of events with both timers is shown in Fig. 7.

timers are shown in Fig. 8. The organization of components in both types is indicated in Fig. 9.

The type A timer consists of one six-switch rotary timer,[17] driven by either a one or a two-hr clock, and two adjustable up-to-one-hr timers[18] linked serially so that they are activated in sequence as shown in Fig. 9. The problem of getting RT (rotary timer), BT (buffer timer), and ET (elution timer) to function in sequence requires that each activate the following one as it times out. Thus each must provide a signal of sufficient duration to start the next, and must then stop, and must not start again until its proper turn. The rotary switch times the main events in the cycle in the A system, the second timer (ET) provides additional flow of

[17] Rotary timer, Model TM6, Eagle Signal, Davenport, IA.

[18] Cycl-Flex timer, Model HP56, Eagle Signal, Davenport, IA.

elution solution at the end of a cycle, and is followed by a buffer wash to regenerate the column which is timed by the buffer timer, BT. With RT set to zero and the master switch on, the motor for RT is not activated, but BT is, allowing the column to be washed before use. (A special switch is provided to activate RT at once however should this be desired.) When the time set on BT has expired, the rotary time RT is activated beginning a cycle. The switches on the rotary timer are all single-pole, double-throw and serve the following functions (the times being indicated as percent of one rotation).

Switch 1. Between 98 and 0% the motor for the rotary timer is turned off, and the elution timer is started.

Switch 2. When the elution timer times out, it provides current through this switch to drive the motor of the RT to the zero position, at which point the BT can activate it.

Switch 3. Selects buffer or sample flow to column.

Switch 4. Selects either buffer or elution solution to the column.

Switch 5. Collects output into *"A"* container vessel.

Switch 6. Collects output into *"U"* collection vessel.

A terminate switch is provided which, at the time of the next sample addition, activates the buffer timer BT instead, and at the end of this additional wash shuts down the entire system.

The B timer system uses four clocks in rotation to control column inputs, while collection is controlled by a separate eight-switch rotary timer which is activated at the completion of sample addition, and which must time out before the next sample addition occurs. The first switch on the rotary timer interrupts current to the timer itself causing it to stop at 0% position, and to operate after it passes the 5% position. Current is provided long enough to get past the 5% position by the timer T3. Thus the rotary timer is started by T3 and stops after one revolution until again energized by T3. The sequence of operation of the timers is shown in Fig. 9. In addition to a termination switch, a counter is provided which allows a preset number of cycles (up to 999) to be completed before the system is shut down automatically.

DISCUSSION

An automatic system, termed a Cyclum, is described which allows affinity or gel chromatography separations to be repeated precisely a large number of times. Since the adjustments of buffer, elution, and sample flow times and duration of fraction collection timers are made in response to the separations actually observed, provision is made for the flexible adjustment of all of these parameters during operation. The objective is to allow amplification of separation techniques so that small amounts of antigen (or antibody) may be used to prepare much larger amounts of antibody (or antigen). In addition the system may be used for

gel filtration separations. When used for ion exchange chromatography where either a freshly packed column is needed for each separation, or where column regeneration is a lengthy procedure, an additional valving system for allowing a series of columns to be used in rotation is required.

Given the Cyclum systems, it is then necessary to work out methods of column preparation and solutions for rapid recycling which do not damage either the columns or the proteins separated. Fortunately the system described allows such development work to be carried out with precise control of all parameters. The development of affinity columns, and methods for the isolation of specific antigens, is described in subsequent papers.

Note added in proof. Advanced versions of these systems are available from Electronucleonics, Inc., Box 451, Oak Ridge, TN 37830.

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