EVALUATION OF AN ASSEMBLY FOR AUTOMATED COLUMN CHROMATOGRAPHY*

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With the introduction of substituted celluloses, cross-linked dextran gels, and a variety of adsorbents for the exchange or exclusion chromatography of macromolecules, techniques for the separation of proteins, nucleic acids, polysaccharides and peptides have been phenomenally advanced. Concomitant with this progress have been technological advances in the formulation and design of equipment capable of detecting the material as it is released from the column as well as separating the eluate into discrete fractions. When ion-exchange chromatography is used for the partition of complex mixtures, selective separation is achieved by appropriate choice of buffers, added either singly in a discontinuous fashion, or, *via* programmed gradients as obtained by a device similar to the "varigrad" of PETERSON AND SOBER[1]. In either case, the pH and conductivity of each fraction should be recorded so that appropriate changes in gradient on rechromatography may be designed to afford the best resolution of the desired fractions. In general, proteins are thermolabile and provision should be made to refrigerate both columns and column eluates.

Specific instrumentation which can perform the aforementioned processes has been available for some time in the form of modular components which are modified by the individual laboratories to suit their specific needs. Recently, the Spinco Division of the Beckman Instrument Company made available a unitized apparatus (Spectrochrom, Model 130) capable of performing all the above-cited functions in a completely automated and integrated fashion. As is often the case in newly-developed apparatus, promise and fruition may be at variance. The purpose of this report is to evaluate the Spectrochrom as a useful and reliable device for the separation of blood and urinary proteins using a variety of chromatographic adsorbents.

METHODS

In general, the chromatographic procedures cited fall into three categories: (a) gradient-programmed separations on DEAE-cellulose**, (b) gel filtration with several types of Sephadex***, and (c) ion-retardation studies with AG-11A8§. This latter material is composed of spherical polymer beads containing paired anionic and

* The statements contained herein are the opinions of the authors and do not reflect any endorsement or recommendation by the Office of the Surgeon General, Department of the Army.
** Obtained from Sigma Chemical Company, St. Louis, Mo.
*** Obtained from Pharmacia Fine Chemicals, Piscataway, N.J.
§ Obtained from Bio-Rad Laboratories, Richmond, Calif.

TABLE I

ADSORBENTS, COLUMN DIMENSIONS, AND BUFFERS USED FOR ELUTION

Column packing	*Number of separations* with the column dimensions (cm):*			*Eluting fluids*
	1.5 × 15	*1.5 × 50*	*1.5 × 150*	
DEAE-cellulose	68	41		TRIS–PO_4 buffers
AG-11A8	2	5		Distilled water
Sephadex G-25			107	PO_4–NaCl buffers, water
Sephadex G-100		22		PO_4–NaCl buffers
Sephadex G-200		49		PO_4–NaCl buffers

* Total number of separations: 294.

cationic exchange sites. The mixtures separated on various columns include whole human serum and fractions thereof, urinary protein extracts, and "protein-free" filtrates of human plasma. Table I indicates the packing material, the average size of the columns employed, the solvents used for elution as well as the number of separations. In all cases, temperature of the column jackets was maintained at 4° and the fraction collector compartment was held at 6–8°. Fractions were collected by drop-count where conducting buffers were used or by timed collection where distilled water was the eluting medium. The absorption of the eluates was monitored at wave lengths varying from 220 through 405 mμ.

Overall views of the apparatus as well as location of some specialized components are shown in Figs. 1 and 2. A schematic representation of the Spectrochrom is shown in Fig. 3. Buffers are pumped from the reservoir bottles, singly or admixed, through the left arm of the dual light path cuvette (2.5 mm and 10 mm) contained

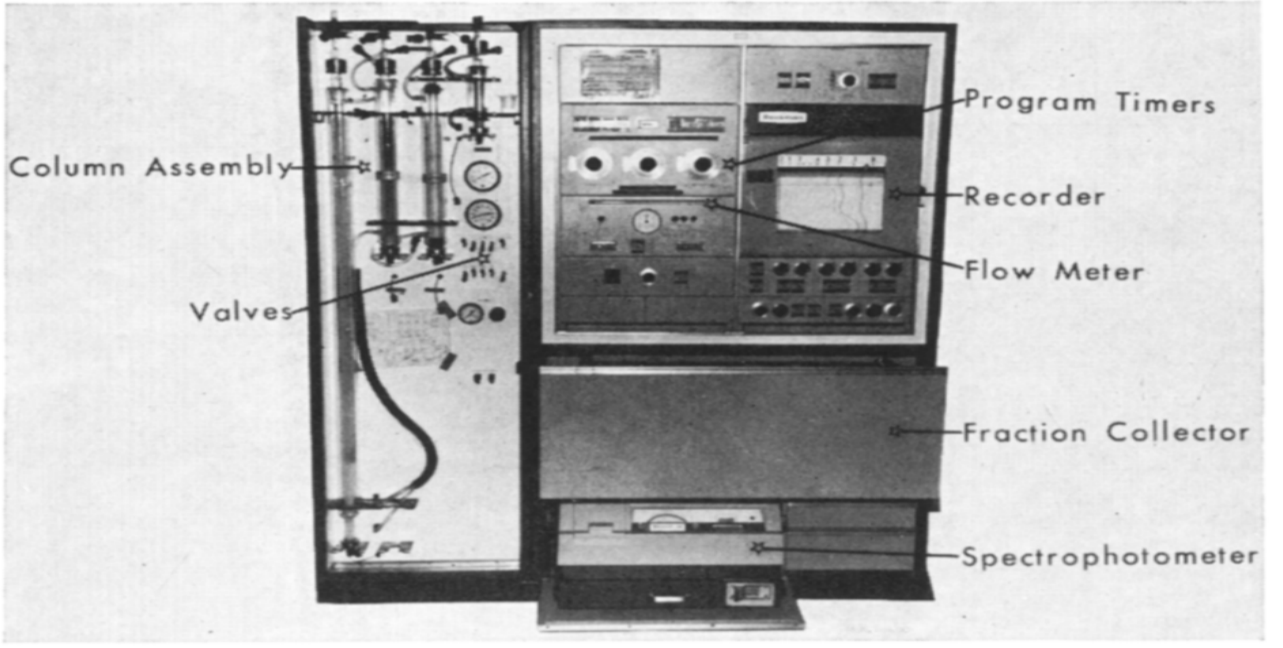


Fig. 1. Spectrochrom, Model 130, front view. Chromatography columns are shown to the left, recording and collecting systems are contained within the right side of the apparatus.

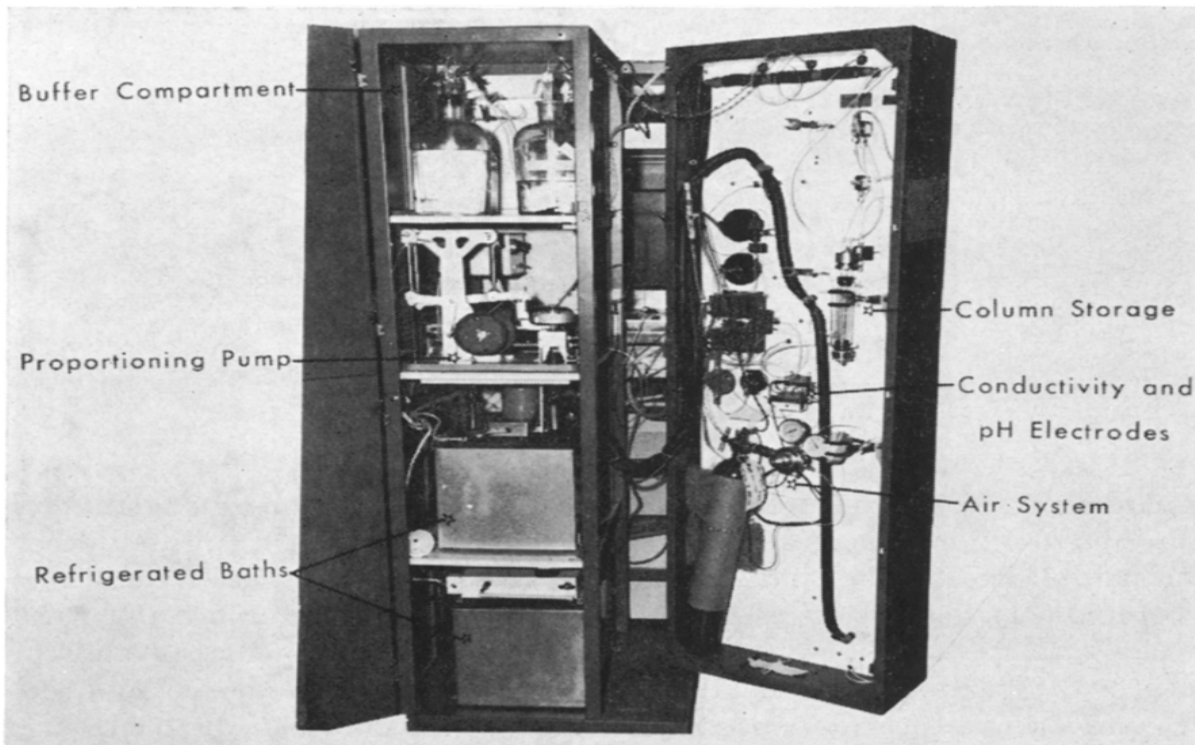
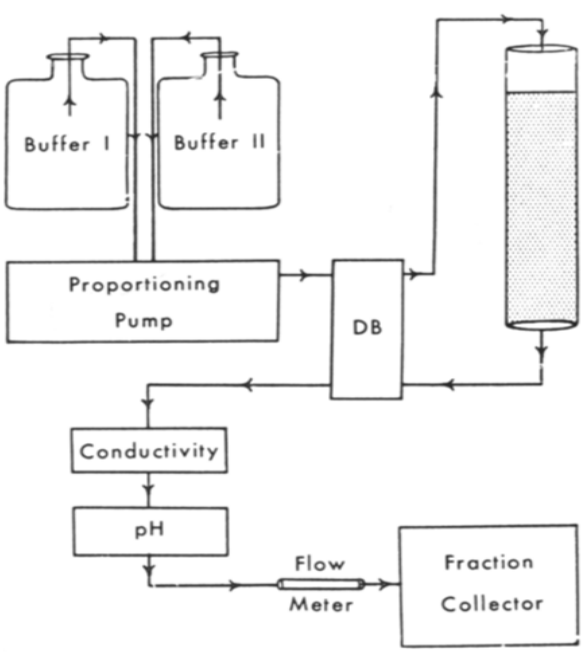


Fig. 2. Spectrochrom, Model 130, left side exposed.



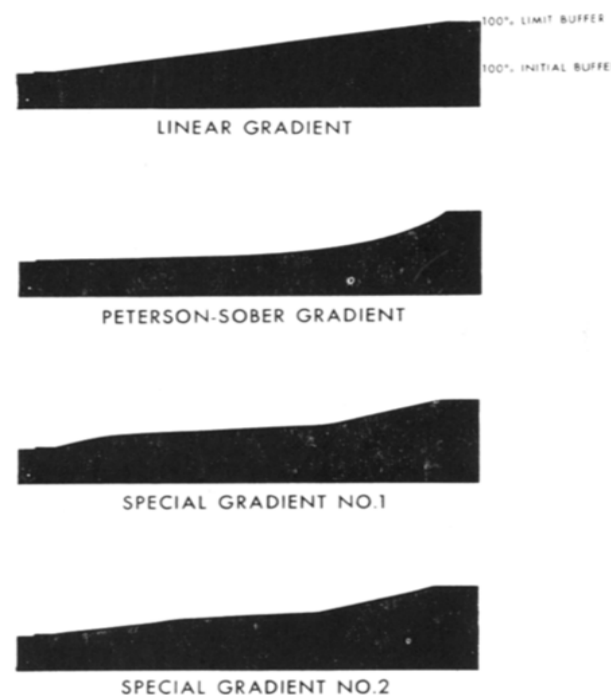


Fig. 3. Schematic view of the flow path within the Spectrochrom.

Fig. 4. Program cams for the proportioning pump.

within the DB Spectrophotometer, on to the top of the chromatographic column. The column outflow is returned to the right side of the cuvette, through the conductivity, pH, and flow meters, and then finally to the fraction collector. Three- and four-way valves permit switching any of the systems in or out of the flow circuit. The monitored variables (pH, conductivity, multiple absorbance) appear as discreet colored dots on a Bristol Recorder. The absorbance is indicated on the Bristol chart paper in a logarithmic rather than a linear fashion. Changes in fraction are indicated by slash marks in the lower portion of the chart. Programming of the buffer flow and shutdown of the apparatus can be accomplished by time-set mechanisms. If desired, programmed changes in the jacket temperature of the columns may be instituted.

When subtle continuous changes of buffer are required, program cams cut to the requisite changes are inserted in the proportioning pump. Control in the amount of each buffer added is by means of a rocker arm whose excursions are limited by the shape of the cam. The linear program cam, another that mimics the PETERSON–SOBER gradient system, and two of our own design are shown in Fig. 4.

RESULTS AND DISCUSSION

Representative chromatographic separations (taken from our routine fractionation programs) have been examined in some detail with view toward evaluation of component function. Included are unsatisfactory records representative of specific malfunctions.

Sephadex G-200 (Fig. 5a)

In this separation, approximately 180 mg of a crude kaolin extract of proteins from post-menopausal urine were dissolved in 10 ml of phosphate–saline, pH 7.4, and applied to a 1.5 × 50 cm column; a flow rate of 18 ml/h was established and 5 ml aliquots were collected[2]. Chart speed was set at 1 in./h and the absorbance at 260, 280 and 405 mμ was recorded at two light paths, along with pH and conductivity of the eluate. The initial buffer was used throughout the elution. This pattern and all subsequent figures were directly photographed from the charts; the blue grid background was faded out to increase the resolution of the spectral absorbance lines. The absorption is indicated on the abscissa, the fractions on the ordinate. As the pattern indicates, the separation and its record are satisfactory.

Sephadex G-100 (Fig. 5b)

One ml of human serum was applied to a 1.5 × 50 cm column with phosphate –saline, pH 7.4, used as the eluting fluid. The absorbance was monitored at 220, 260 and 280 mμ at a flow rate of 27 ml/h. In this study, the fraction collector was bypassed and no aliquots were collected. The column and monitoring apparatus worked quite well as evidenced by the detailed resolution of the components.

Sephadex G-25 (Fig. 5c)

The protein-free filtrate from 5 ml of plasma (prepared by addition of perchloric acid, then neutralization with potassium hydroxide) was applied to a 1.5 × 150 cm column. The absorbance of the distilled water eluate (38 ml/h, 5 ml aliquots) was followed at 225, 260 and 280 mμ. The use of multiple absorbance settings permits the

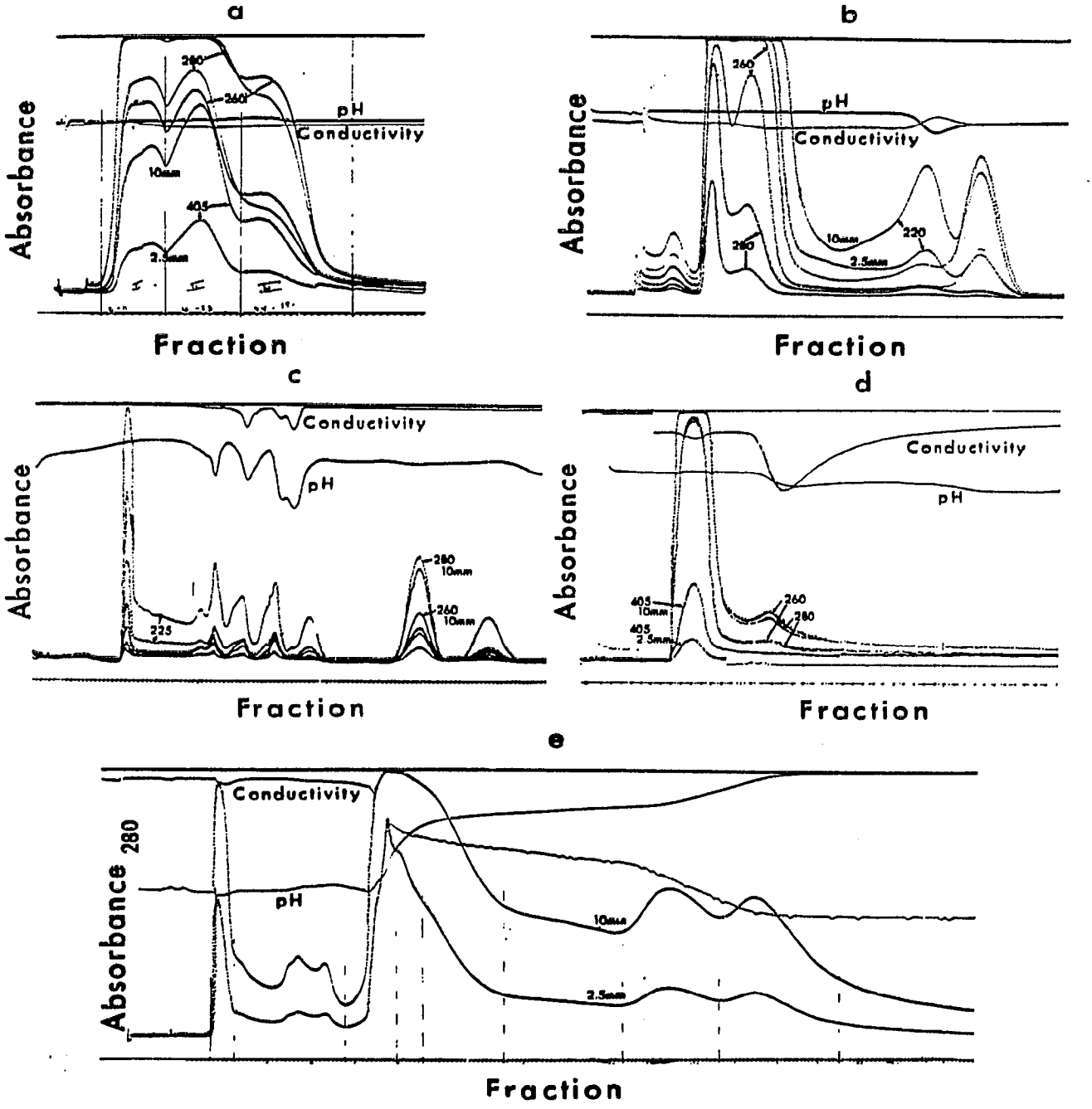


Fig. 5. Representative separations on varying chromatographic adsorbents: (a) Sephadex G-200; (b) Sephadex G-100; (c) Sephadex G-25; (d) AG-11A8; and (e) DEAE-cellulose.

detection of small amounts of material and additionally aids in the identification of general classes of materials separated by virtue of their relative absorption at selected wave lengths. As the salts originally contained in the sample are released from the column, associated changes in the pH and conductivity of the eluate are shown on the chart.

AG-11A8 (Fig. 5d)

The material is a polymer with weak exchange properties and is commonly used for desalting protein solutions (ion retardation). In this separation, 2 ml of human plasma, diluted to 10 ml with saline, were applied to a 1.5 × 50 cm column and elution performed with distilled water (flow rate, 32 ml/h, 10 ml aliquots collected). The column eluate was monitored at 260, 280 and 405 mμ. As noted from the pattern, an increase in conductivity appears after the major protein peak has passed through the column and would correspond to the release of retarded salts from the column.

DEAE-cellulose (Fig. 5e)

All the separations previously discussed represented single buffer elution and did not fully utilize the proportioning pump. In this study, 480 mg of urinary protein (identical to that applied to the G-200 column of Fig. 5a) were dissolved in 30 ml of a 0.04 *M* TRIS–0.005 *M* PO_4, pH 8.6 buffer, and then applied to a 1.5 × 50 cm DEAE-cellulose column[3]. The initial buffer was maintained until fraction 21 (210 ml), then the program device was set in operation and a limit buffer consisting of 0.5 *M* TRIS–PO_4, pH 4.0, was added in a gradual and predetermined fashion. The gradient (Special No. 1) used is shown in Fig. 4. To reinforce the absorbance recording all wave length calibrations were set at 280 mμ. Since cellulose columns permit high flow rates, elution was done at 120 ml/h. The change in pH as well as variation in conductivity are clearly shown on the chart. Although the pH changes, as shown, are accurate and in accord with the programmed gradient, the conductivity determinations are at variance from true values. This departure will be discussed in a later section.

Figs. 6a and 6b are illustrative of non-satisfactory records which result from some malfunction in the system.

Chromatographic "failures"

Fig. 6. illustrates faulty records obtained with protein mixtures separated on columns of Sephadex G-200 and DEAE-cellulose, respectively. The upper recording is a separation identical to that shown in Fig. 5a. In this case, the completely erratic pattern resulted from air bubbles trapped within the flow-through cuvette. This has occurred more often with Sephadex columns and may, in part, be related to slow flow rates and non-refrigeration of the lines leading from the chilled column to the fraction collector. Bubbles formed in the eluate upon rewarming can exceed the bubble trap capacity, lodge in the cuvette, and produce an illegible record. Fig. 6b shows a urinary protein mixture separated on DEAE-cellulose in a manner identical to that previously described (Fig. 5e). In this case, the change in absorbance was not due to eluted protein, but faulty operation of the recording spectrophotometer occasioned by misalignment of the optical system as well as changes in the automatic slit control. In many of our early separations, a number of poor records were obtained

due to faulty spectrophotometer operation. Change of the spectrophotometer with incorporation of suggested changes in the replacement considerably reduced the failure rate. Despite the detailed instructions given in the manual, optical alignment is best left to the professional.

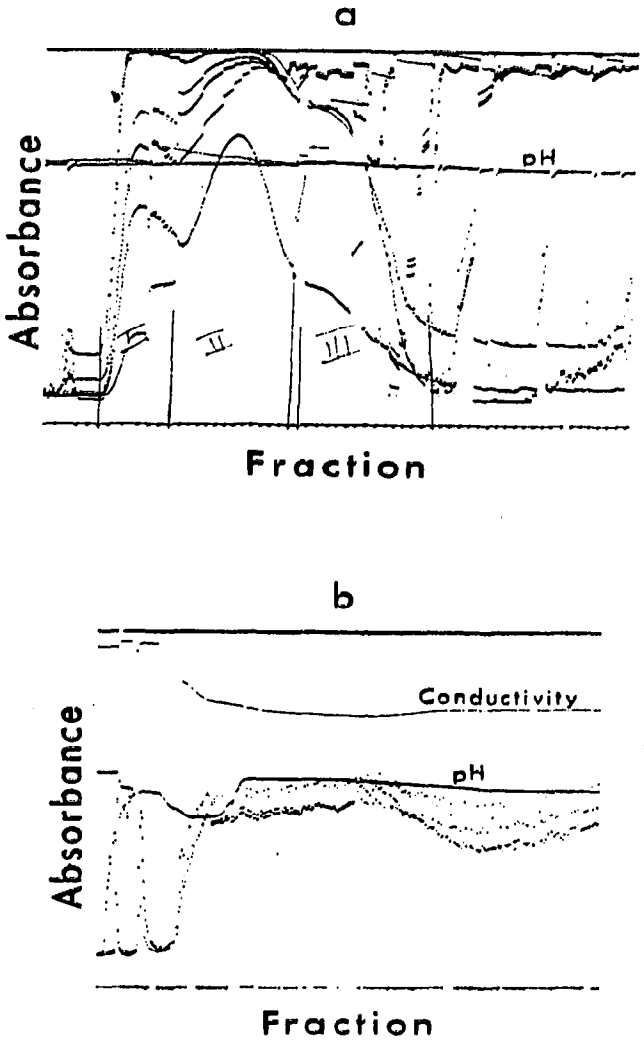


Fig. 6. Representative recordings indicating malfunction of the apparatus: (a) Sephadex G-200 and (b) DEAE-cellulose.

Summary of the major chromatographic separations obtained to date

The figures shown in Table I represent the number of separations performed under the conditions stated. Apart from the 294 listed, this laboratory has done an additional fifty runs of varying type using other adsorbents and column sizes. Initially (the first fifty separations) the failure rate was in excess of 25%, but added familiarity with the apparatus as well as revisions in various components have reduced this rate to less than 10%. In the larger number of these "failures", malfunction was evidenced in the recording, but the separations were obtained as programmed and the fractions could be read manually. The series reviewed were done over a period of 20 months.

Evaluation of the components

Some of the strengths and weaknesses of this automated system are apparent from the preceding discussion. In some cases, entirely new components were specifically designed for this apparatus; in other cases, they are standard commercial items minimally altered to suit the new equipment. The chromatography columns are well made, easy to pack, clean, and use. However, the metal screw at the top of the column can become frozen when salt-encrusted and accordingly difficult to remove. This problem might be avoided if holes were drilled to accommodate a spanner-type wrench or if nylon or teflon were substituted for the metal parts. Vacuum jackets are a necessity since the initial jackets supplied with the unit developed excessive condensation. Column cooling, as well as refrigeration of the fraction collector, worked well, although connection of the cold-water circulating lines to the column jackets is such that change of columns cannot be easily accomplished without fluid loss. In addition, provision should be made for refrigerating the buffer compartment as well as insulating the lines leading throughout the system.

For single buffer operation, the proportioning pump has been evaluated at delivery volumes ranging from 12 to 120 ml/h and found to be extremely reproducible. When gradients elutions were employed, the pump functioned well, and apparently accomplished the work of the "Varigrad" in a far simpler fashion. Care should be taken to bleed the delivery pistons free of any air to prevent admixture of buffer.

Although the response of the electrode to change in pH is somewhat slow, the values obtained on the chart were comparable to the measurements obtained with a laboratory meter. The manipulations required to change the electrodes are somewhat difficult, but changes are not often necessary. The conductivity meter, purported to record in a linear fashion, does not perform as described. Although changes in conductivity are clearly indicated, they do not represent actual values. However, the curve shape is useful in following gradient changes. To our knowledge, this component is undergoing complete re-design.

As indicated earlier, the largest number of our initial problems were encountered with the spectrophotometer. A complete change of unit in addition to the substitution and relocation of a heat-resistant capacitor did away with much of the early instability. The dual-path flow-through cell is quite ingenious and immeasurably adds to versatility of the apparatus. If both visible and U.V. ranges are scanned in a single run, adjustment of condensing mirror and alignment of the optical system are critical. Moreover, these adjustments should be periodically checked.

The fraction collector is a standard unit which has been directly incorporated into the present apparatus. Although some initial difficulty was encountered as the result of faulty soldering, the collector has worked well on both drop and timed collections. The volumetric collecting head has been used with another fraction collector, and performed satisfactorily. Since drop and volume systems depend on electrically conducting fluids, it is necessary to use timed collections for all distilled water operations.

Systems which permit automatic, unattended operation are those which lend greatest appeal to laboratories doing a large number of chromatographic separations. These have functioned reliably and well, permitting buffer changes, specified shutdowns, etc.

The Spectrochrom uses a multi-point Bristol recorder, which is quite easy to

ink, possesses good stability and marks in a clear manner. Due to the placement of the door, gear changing for paper speed control is very awkward. It is our understanding that a recorder attachment is available from the Bristol Company which reduces chart-speed changes to control of a single knob. We would recommend that it be incorporated into the present apparatus.

The air supply system is extremely useful for rapid application of samples on to cellulose columns as well as providing pressure for the regeneration buffers. This permits preparation of unused columns during standard operations. The distilled water flush system is also air-controlled. The bubble flowmeter functions well and adds to the general utility of the apparatus.

On the basis of what has been described, we feel that a completely automated device such as the Spectrochrom is an asset to laboratories doing a large number of chromatographic separations. The limiting features of this particular apparatus should be amenable to repair or re-design. At the present time, the machine is kept operative for 60–70 hours a week. Some of the initial difficulty encountered would have been obviated had the apparatus been factory tested for a longer period prior to shipment. Additionally, the instrument should be placed under regular service so that appropriate preventive maintenance can be instituted. The cleaning of pump pistons, adjustments of the spectrophotometer and general overhaul are better done by service engineers. However, the techniques associated with actual use of the apparatus are not difficult and training of personnel is easily accomplished.

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SUMMARY

An automated apparatus for column chromatography (Spectrochrom, Model 130) has been evaluated for performance and reliability. Although certain deficiencies exist, these would appear to be capable of correction. This apparatus has permitted us to greatly increase our scope in preparative and analytical column chromatography, and the feasibility of completely automating the techniques would appear to be justified.

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