High-Pressure Column Chromatography. I. Design of Apparatus and Separation of Bases, Nucleosides, and Nucleotides [1]

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SUMMARY

Theoretical studies suggest that the resolution obtained with ion exchange columns increases when the size of the ion exchange resin particles and the diameter of the column are decreased. In the development of precise, automated microanalytical systems for the analysis of complex biological mixtures, these, as well as other parameters, must be explored. Very high pressures are required to obtain adequate flow rates through fine resin beds. An experimental system capable of performing separations at 4000 lb/in² (psig) is described and its application to the separation of bases, nucleosides, and related materials is discussed. The system consists of a high-pressure variable volume pump, a steel column, and a spectrophotometric monitoring system. Elutions of base and nucleoside mixtures and simulated ribonucleic acid hydrolysates have been performed with acetate buffers. At high pressure, superior resolution is obtained and elution times are shorter than with previously described systems. In addition, fractionated sample components elute in small volumes of buffer.—Nat Cancer Inst Monogr 21: 431–440, 1966.

FOLLOWING THE introduction of the automated amino acid analyzer (*1*), automatic systems have been developed for the analysis of other classes of substances, including nucleotides and nucleotide derivatives (*2*, *3*) and simple carbohydrates (*4*), on ion exchange column. In these instances, the sample sizes, though small, are still intermediate between those used in laboratory-scale preparative columns and those required for truly ultramicroanalytical procedures. For many research purposes, including clinical survey studies, no preparative requirements exist, and interest shifts in the direction of speed of analysis, precision, decrease in sample size, and miniaturization of the purely analytical equipment. Refinements permitting considerable reduction in sample mass and analysis time have been described for the amino acid analyzer (*5*, *6*) and have been studied during the development of the carbohydrate analyzer

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[2] Operated for the U.S. Atomic Energy Commission by the Nuclear Division of Union Carbide Corporation.

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(*4*). Of the many complex problems that must be solved in the development of miniature ion exchange analyzers, that of obtaining high-resolution separations in a short time has been examined first.

An expression for determination of the effective theoretical plate height, H, of a column in elution development of trace quantities is (*7*):

$$H = 1.64\,r_o + \frac{0.14 + r_o^2 v}{\overline{D}(\lambda_i'' + \beta)^2} + \frac{0.266\,\lambda_i''^2 r_o^2 v}{D(1 + 70\,r_o v)(\lambda_i'' + \beta)^2} + \frac{D\beta\sqrt{2}}{v}$$

where

H = effective theoretical plate height
r_o = particle radius
v = linear flow rate (ml cm^{-2} min^{-1})
D = diffusion coefficient in interstitial liquid
$\overline{D}$ = interdiffusion coefficient in the ion exchanger
λ_i'' = column distribution ratio
β = fractional void volume in the resin bed

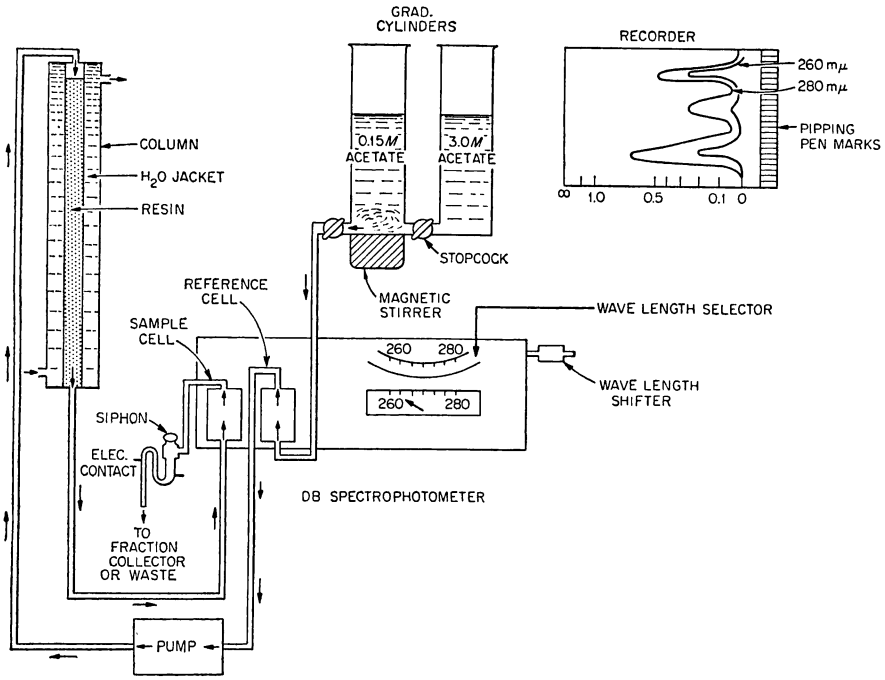
The four terms of the equation express the effects of particle size, slow particle diffusion, slow film diffusion, and longitudinal diffusion, respectively. The best separations are obtained when the value of each term is at a minimum and, thus, the number of theoretical plates for a given column is at a maximum. For uniform operating conditions a reduction in exchanger particle size, r_o, reduces the value of the first three expressions. In practice, the value of the particle size effect is usually doubled to allow for imperfect column packing (*7*). Such compensation can be reduced by use of spherical resins of uniform particle size and by reduction of the diameter of the column. When separations are optimal, band widths are minimal and flow rates may be increased to reduce the time required for an analysis. In addition, increased recorded peak heights result in a net over-all increase in the sensitivity of a system.

Reductions in column diameter and resin particle size increase flow resistance. Higher pressures are required to maintain flow rates, and such pressures can surpass the capabilities of systems using glass columns. The purpose of this work has been to examine the performance and define the problems of chromatographic systems that operate at high pressures. For comparison with our existing conventional system (*3*) and for elimination of the development of an eluate processing procedure, the high-pressure modification was applied to the separation of bases and nucleosides. Elution principles and the spectrophotometric monitoring system have been described previously (*2*, *3*).

DESIGN AND OPERATION

Design

The analytical system, diagrammed in text-figure 1 and shown in figure 1, consists of (*a*) a system to form concentration gradients and to



TEXT-FIGURE 1.—Diagram of an experimental system for the evaluation of high-pressure elution techniques.

deaerate the elution buffer, (*b*) a pump to deliver the buffer to the column, (*c*) a metal column to support an anion exchange resin bed, and (*d*) a spectrophotometric system to monitor the ultraviolet absorbance of the eluate at two wavelengths.

Linear or convex gradients are formed by using connected cylinders, with the terminal cylinder heated to deaerate the buffer. To minimize evaporation from this cylinder, a drop-shaped condenser is placed in the top opening. The cylinders are interconnected through capillary stopcocks, and the gradient system is connected to the inlet of the pump through a 3-way stopcock, which allows the system to be drained.

Buffer is delivered to the column by a diaphragm pump [3] capable of delivering liquids at pressures to 5000 lb/in^2 (psig) at adjustable rates. Influent pressure is indicated by a gauge [4] having wetted parts of stainless steel and a range of 0 to 5000 lb/in^2. The gauge serves as a damper and reduces undesirable hydraulic shock to a minimum. The pump, gauge, and column are joined by ¼ inch O.D. by 1/16 inch I.D. tubing and appropriate tube fittings of 316 stainless steel. To prevent the back flow of particulate material into the pump, a valve is incorporated in the line connecting the pump to the column.

[3] Laboratory Feed Pump Model LP10 with 5 mm plunger and 30 : 1 gear ratio. Whitey Research Tool Co., 5525 Marshall St., Oakland, Calif.

[4] Acragage with shockstop and capillary bleed. Robertshaw Controls Co., Fulton Sylphon Division, Knoxville, Tenn.

The column is pictured in figure 2 and diagrammed in text-figure 2. The main column is fabricated from ⅜ inch O.D. by ⅛ inch I.D. 316 stainless steel. The fitting [5] at the top of the column allows direct access without disconnecting the buffer inlet. The resin is supported on a sintered stainless-steel disc of 5 μ porosity which is sealed to the column by an O-ring. Eluate passes from the column through hypodermic tubing and Teflon tubing (AWG 22). A metal water jacket is incorporated into the column assembly by the use of heat exchanger tube fittings. Heated water is supplied to the column jacket from a circulating constant temperature water bath, passes from the column to the water jacket of the terminal gradient cylinder (not shown), and returns to the bath.

The absorbancy of the column eluate was monitored by a Beckman DB spectrophotometer at 260 and 280 mμ and recorded.

Operation

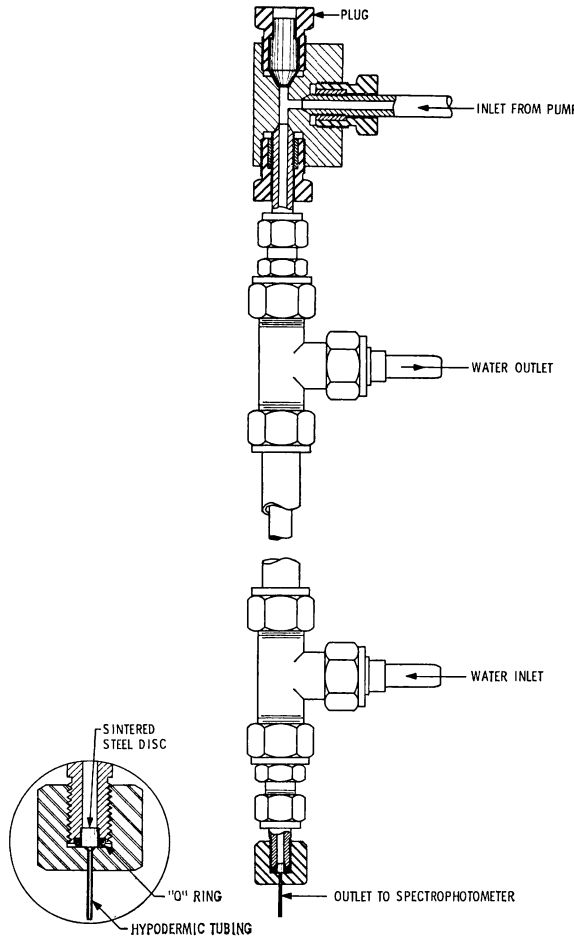
Two separate lots of Dowex 1-×8 anion exchange resin have been used in the evaluation of the system. Initially, a portion of a pilot plant production from the manufacturer [6] was fractionated hydraulically to yield material with a mean particle size of 10 μ and a size range for 90 percent of the particles of 9 μ. Subsequently, an analytical-grade fractionated resin was obtained from a commercial source.[7] The latter exchanger possessed a settling rate of 0.27 cm per minute in water at room temperature, a mean particle size of 13 μ, and a size range of 7 to 28 μ for 90 percent of the particles. Before use, a resin was washed with 6 N HCl, converted to the hydroxyl form with NaOH, and then to the acetate form with sodium acetate buffer. It was packed in the column in increments from a 50 percent slurry in stock acetate buffer, by use of the buffer pump.

Separations have been performed with sodium acetate buffers at *p*H's from 4.1 to 4.6. Stock buffer was obtained by mixing 3 M sodium acetate and 3 M acetic acid in correct proportions to obtain the desired *p*H, and elution buffers were diluted from this solution.

Two methods of placing a sample on the resin have been used. In one procedure, the sample was absorbed on a bit of cotton and carefully tamped on the surface of the exchanger. The remainder of the volume was filled with buffer, the column sealed, and the elution begun. In the alternate procedure, buffer was removed from the column by aspiration and the sample, in a volume of 75 μl or less, was placed directly on the resin surface. Finally, the column was sealed and elution begun.

Two standard solutions, 10 mM with respect to each component except guanine, which is 1.0 mM, have been used. One contained cytosine, cytidine, uracil, uridine, thymidine, inosine, adenine, adenosine, guanine, and guanosine. The other, simulating an alkaline ribonucleic acid (RNA) hydrolysate, contained the mixed 2′ and 3′ monophosphate ester isomers of cytidine, adenosine, and guanosine, and the 3′ ester of uridine.

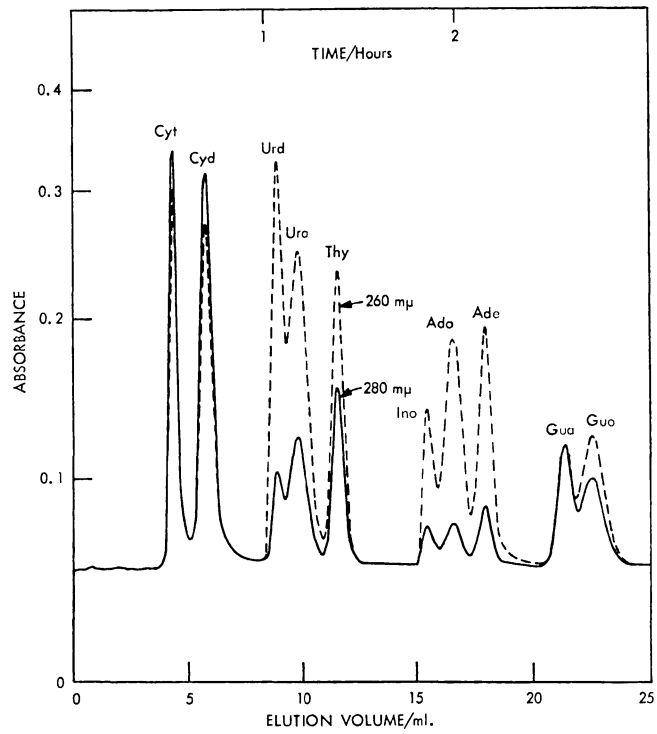
[5] Catalogue No. 45-4321, American Instrument Co., Inc., Silver Spring, Md.
[6] Lot No. 5893-35, Dow Chemical Co., Midland, Mich.
[7] Lot No. B2082, Bio-Rad Laboratories, 32d and Griffin Ave., Richmond, Calif.



TEXT-FIGURE 2.—High-pressure ion exchange column. Except where noted in the text, the assembly is fabricated from readily available tube fittings and accessory components. The sintered disc is machined from a larger disc obtained from Crawford Fitting Company, Cleveland, Ohio.

RESULTS AND DISCUSSION

For acceptable separations of bases and nucleosides in this system, it has been necessary to elute with both *p*H and concentration gradients at low flow rates and to establish a temperature program. A chromatogram obtained from an elution involving variation of these parameters is shown in text-figure 3. Elution was accomplished with buffers of low concentration, and the *p*H change involved in this elution should not affect molar absorbancy values. As a result of using a small-bore column, eluted peaks were contained in minimal amounts of buffer. For example, cytosine eluted in 1.26 ml and guanosine in 2.52 ml of liquid. An analysis was completed in less than 3 hours; a comparable elution requires 6.5



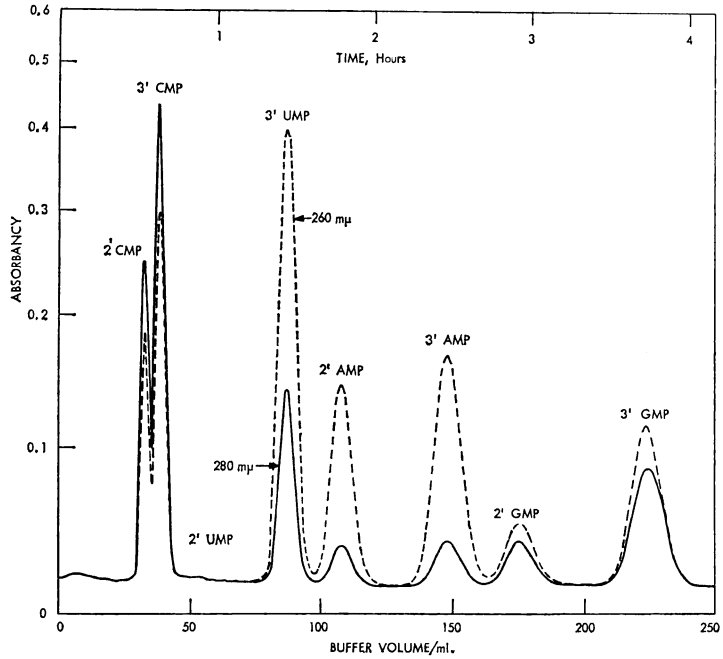
TEXT-FIGURE 3.—Separation of bases and ribonucleosides with a high-pressure elution system. At 72 minutes from the start of the analysis, the temperature was shifted from 30 to 50° C at 4° C per minute. The elution gradient was composed of 40 ml of 1.5×10^{-3} M (acetate ion) buffer at *p*H 4.4 in the mixing cylinder and 40 ml of 3×10^{-3} M (acetate ion) buffer at *p*H 3.9 in the back cylinder. Sample was 0.25 μmole of each component. Column diameter and length were, respectively, 0.125 inch and 36.0 inches.

hours in the system described previously (*3*). Although separation of eluted peaks was not ideal, quantitation was possible.

The analysis of a simulated RNA hydrolysate is shown in text-figures 4 and 5. When unusual components are anticipated or separation of isomers is desired, the 4-hour elution can be used, but when rapid analyses are desired, the 2-hour program is applicable. Temperature and *p*H programming were not required for these elutions.

With the present system, separations have been performed at pressures to 4000 lb/in^2. Presently, the capability of the pump limits the pressure at which the system may be operated and, consequently, the minimal bead size of the resin that may be used.

Introducing a sample into the high-pressure column remains a problem. When the sample was sorbed on cotton, total transfer to the resin was not obtained. The layering technique has been satisfactory, but sample



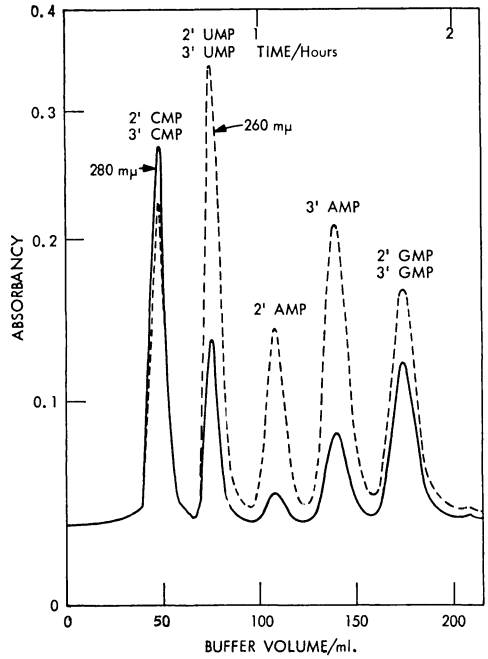
TEXT-FIGURE 4.—Analysis of a simulated ribonucleic acid hydrolysate by normal elution procedure. Gradients, expressed as acetate ion, were 120 ml each of 0.05 M and 1.0 M buffer at *p*H 4.1. Sample sizes were 0.5 μM of each component. Elution temperature was 45° C.

size is severely limited. Ultimately, a variable volume sample injection system with very high reproducibility would alleviate this problem.

As found in other chromatographic systems, the quality of resolution is limited by the performance of the ion exchanger. The mechanical design of the system can only exploit the properties inherent in the resin. Of several resins examined, only lot B-2082 gave satisfactory resolution in this system. However, with careful control during manufacture, materials may be produced that are adapted to specific separations.

Reduced elution times permit increasing the number of analyses that may be performed in a given time. Shorter columns mean smaller systems and reduced requirement for expensive—perhaps especially synthesized—resins. Eluted peaks appearing in minimal volumes required reduced postelution processing for recovery. Additional refinements may make the system a useful laboratory tool for both analysis and studies on the effect of particle size on resolution.

Cell fractions obtained by zonal centrifugation with the combined rate-isopycnic banding technique (*8*) do not yield large quantities of each of the many fractions seen. The analysis of these fractions for nucleic acids and nucleic acid derivatives will be greatly facilitated by the development of automated analytical systems requiring very small samples.



TEXT-FIGURE 5.—Analysis of a simulated ribonucleic acid hydrolysate by accelerated elution procedure. Gradients, expressed as acetate ion, were 100 ml each of 0.15 M and 0.30 M buffer at *p*H 4.4. Sample sizes were 0.5 μM of each component. Elution temperature was 45° C.

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PLATES

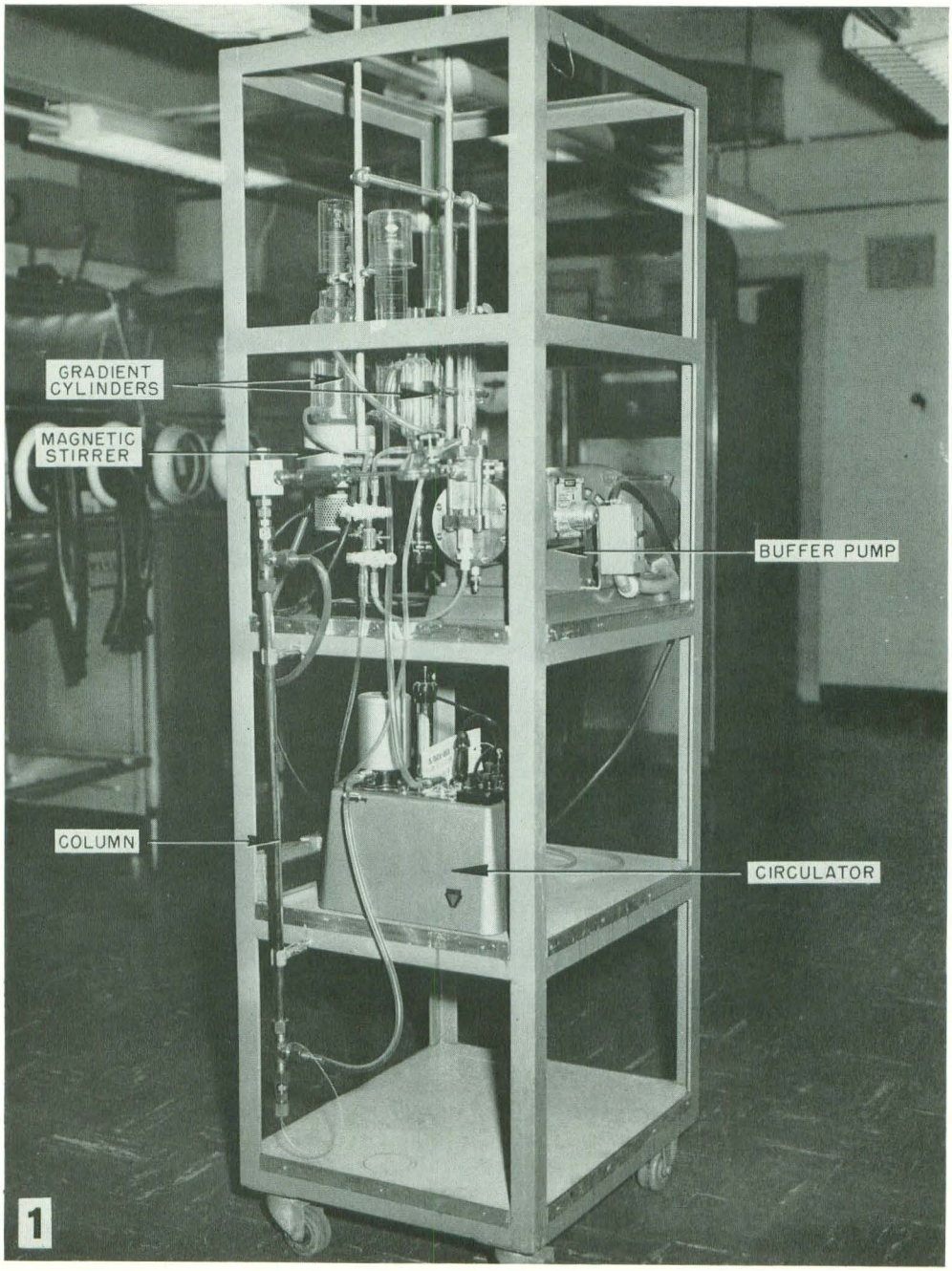

FIGURE 1.—The column module of the high pressure system. Eluate is conducted from the column to the spectrophotometric column through capillary Teflon tubing. The monitor module is not shown but has been described elsewhere (*2*).

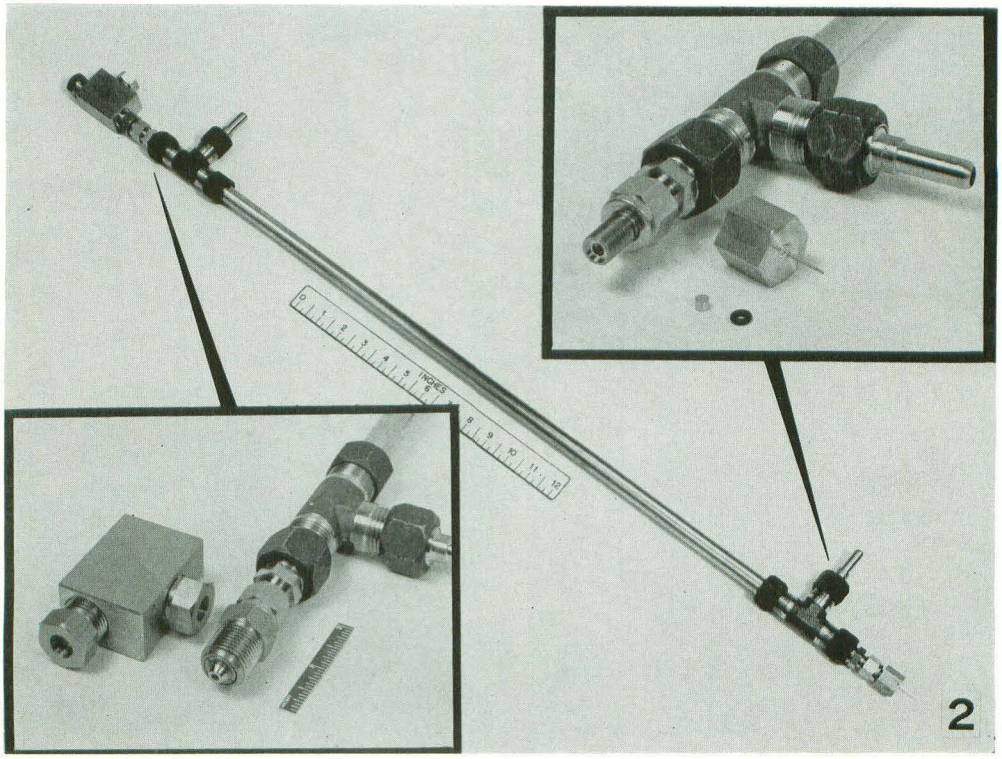

FIGURE 2.—Column used for the study of high-pressure elution techniques.