A DEMOUNTABLE TUBE* FOR LARGE-SCALE CHROMATOGRAPHY AND ITS APPLICATION TO THE ISOLATION OF HEMOGLOBIN A_{Ic}

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For chemical investigations of the structure of the minor hemoglobin component A_{Ic} (ref. 1) which occurs to the extent of 5 to 7% in normal adult human hemoglobin[2,3], it has been necessary to isolate several grams of material. Although this amount may be isolated by repeated small-scale chromatograms, a large-scale chromatographic column (10 × 100 cm) has been successfully operated with a load of 50 g of hemoglobin. The construction and use of this chromatographic apparatus is described herewith; some results of the chemical investigations of hemoglobin A_{Ic} have been presented in part[4] and will be described in detail elsewhere[5].

CONSTRUCTION OF THE CHROMATOGRAPHIC TUBE

A photograph of the filled chromatographic tube, its supports and accessories is shown in Fig. 1. Because of the chromatographic properties of hemoglobin A_{Ic} under the specific conditions employed, the tube was constructed in two approximately equal lengths which were held together with a pipe flange. In this way, hemoglobin A_{Ic} could be developed until it alone was present in the lower half of the column. The two parts could then be separated, and the hemoglobin could be rapidly eluted from each by altering the conditions. (In other applications, a different proportion of lengths may be advantageous.)

The upper half A (Fig. 1) was constructed of 10-cm (I.D.) Pyrex tubing which was fused to a 4-in. Pyrex pipe joint (Corning Glass Works, Corning, N.Y.). The water jacket was of such dimensions that the water annulus was 6 mm thick; the water inlet is E (Fig. 1). The socket joint M at the top was 65/40 in size.

The lower half B was constructed in similar fashion. To support the resin, a coarse sintered funnel K was fused into the tube. An 18/9 ball joint L at the bottom served as a connection to direct the effluent to the desired place.

This apparatus was designed for easy portability, and the pipe flange which connected the two sections was incorporated into the support. A triangular pressboard base G (approx. 65 cm on a side) with swivel wheels H supported the three legs of the steel tripod F. Each leg was bolted directly to the lower half of the pipe flange D (Pyrex pipe to Pyrex pipe flange, 4-in. size, Style 1, aluminum, with inserts,

* The chromatographic *tube* should be distinguished from the chromatographic *column*. The former contains the solid support or *column* on which the chromatographic separation takes place.

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Corning Glass Works, Corning, New York). Part B rested on the pipe flange D and was further supported by the rubber covered metal ring I with extensions 120° apart that were connected to the turnbuckles J for adjusting the position of I. A Koroseal gasket C (Type R-3 for 4-in. flange, Corning Glass Works, Corning, N.Y.) separated the two parts of the tube and provided a leak-proof seal as well as a cushion to prevent breakage. Six stainless steel bolts held the two halves of the flange and, hence, of the tube together.

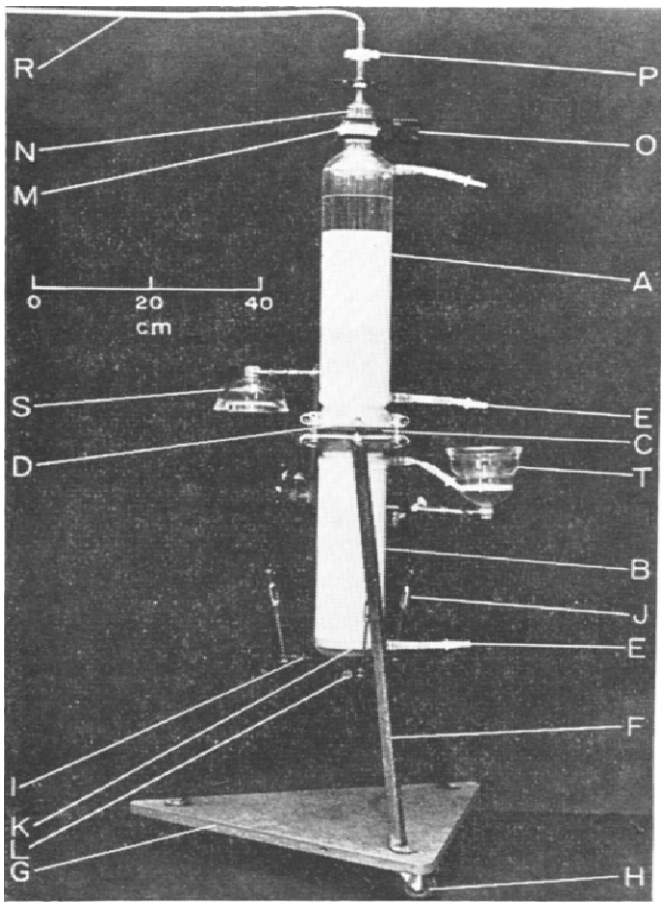

Fig. 1. The 10 × 100 cm chromatographic tube, its supports and accessories.

Developer was directed to the chromatogram through tubing R, Teflon stopcock P (with an 18/9 ball joint), and adapter N (ball joint 65/40, socket joint 18/9). A pressure clamp O held the parts of the joint together. A thin silicone rubber gasket was placed between the parts of a large joint such as M in order to prevent leakage.

Parts S and T are accessories that were used after the two sections of the column had been separated at the end of the chromatogram. In Fig. 1, they are supported by clamps for illustrative purposes only. Part S, thus, is an adapter of 4-in. Pyrex pipe and an 18/9 socket joint that is connected to B in order to direct developer through this portion of the column alone. Likewise, T is an adapter of 4-in. Pyrex pipe, a coarse sintered disc, and an 18/9 ball joint which, when it was attached to A, permitted this section to be eluted separately.

OTHER EQUIPMENT

For chromatography on this scale, several items of equipment are necessary for handling large volumes of ion exchange resin and developer. As a reservoir for developer, a 13-gallon (56-l) aspirator bottle (Q, Fig. 2) was used (Polyethylene aspirator bottle, 13-gallon capacity, with tubulation, Nalge No. 2302, The Nalge Co., Inc., Rochester, N.Y.). Two other bottles of the same size (Polyethylene carboy, 13-gallon capacity, with hand-grips, Nalge No. 2210) were required for preparation and storage of developer. Small carts were convenient for the transportation of the filled carboys In addition, a polyethylene tank (rectangular polyethylene tank, 10-gallon capacity, Nalge No. 11000, with cover No. 11003) was utilized for the preparation of ion exchange resin before the column was packed.

In the application of this apparatus to the isolation of hemoglobin A_{Ic}, the developer contained potassium cyanide (0.6 g/l) which at neutral pH is present in solution as hydrogen cyanide. In a closed cold room without addition of fresh air, a lethal concentration of cyanide could easily develop. In the interest of safety, it is advisable to monitor the concentration of hydrogen cyanide with a detector kit for hydrogen cyanide gas (No. DH 73493) which is available from the Mine Safety Appliance Company (201 N. Braddock Ave., Pittsburg 8, Pa.). For use in an emergency, a positive pressure type gas mask and amyl nitrite should be readily available. When all containers were carefully closed, the concentration was easily kept to a safe level of 2 p.p.m.

PROCEDURE

The procedure differs from that previously described[1,2,4,6] essentially only in the scale. The following description, then, is concerned only with those factors that must be considered because of the increased scale.

Approximately 15 lb. of (wet) resin were required for a 10 × 100 cm column. Bio-Rex 70 (which is equivalent to IRC-50) was purchased from Bio-Rad Laboratories, Richmond, Calif. in actual wet mesh size 200–325. The resin in the sodium form was suspended by vigorous stirring in 10 times its settled volume of distilled water and then allowed to settle for 30 min after which time the supernatant fluid with suspended fine particles was removed. Although the amount of fine material was small, three settlings were made. The resin was then suspended in twice its volume of the buffer which was to be used for equilibrating the column and was adjusted to the pH of the equilibrating buffer with phosphoric acid. The column was poured from this suspension at room temperature in a single section: as the resin in the tube settled under gravity and the liquid drained from the bottom, additional suspension was added.

Fig. 2 shows schematically a convenient arrangement for handling the necessarily large volumes of developer. The 56-l aspirator bottle Q should be placed at maximum height to take advantage of the hydrostatic pressure thus produced. The tubing R (Figs. 1 and 2) led directly to the column from the tubulation at U. A stopcock and an inlet tube with an 18/9 socket joint V passed through the rubber stopper in the neck of the bottle. A pressure collar retained the rubber stopper in the neck when the bottle was under pressure. A small centrifugal pump was used to

transfer developer to reservoir Q from the carboy in which it was mixed. During this operation, the line W from the pump was connected to V. During equilibration and development, the line X from the tank of compressed nitrogen was attached to V and the stopcock was closed.

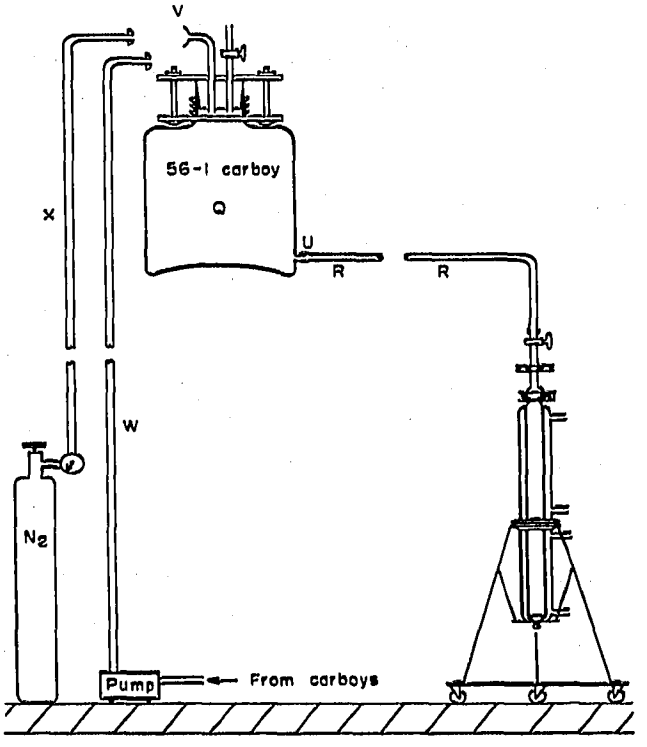

Fig. 2. Arrangement of apparatus.

Before use, the column was equilibrated (in this application with Developer No. 6 (ref. 7)) in the cold room at 2° with 160 l of buffer. With a hydrostatic head of approximately 3 m from the reservoir to the bottom of the column and the additional application of 0.5 atm of pressure from compressed nitrogen, the developer flowed through the column at approximately 2 l/h. (Compressed gas pressure should be applied to polyethylene carboys with caution. The application of pressure to the carboy that was used caused the concave bottom to become convex and tended to tip the carboy over.)

After the column had been equilibrated, the buffer was removed from the top, and the sample (about 50 g of adult carbon monoxyhemoglobin from 1 pint of blood in 500 ml of Developer No. 6 after thorough dialysis) was poured onto the resin bed and stirred into the resin to a depth of 5 cm to give an even boundary between the stirred and unstirred portions. After the sample had entered the column, the sides of the tube were rinsed carefully three times with about 25 ml of buffer. Finally, the remainder of the tube was filled with buffer, and development was carried out at approximately 2 l/h.

With Developer No. 6, "non-heme proteins" and the minor components A_{Ia} and A_{Ib} (ref. 1) separate from and move down the column ahead of A_{Ic}. Because the isolation of A_{Ic} alone was of interest, the effluent which contained these faster moving components was directed to a drain and diluted with a stream of water to reduce

the cyanide concentration. After approximately 36 h of development (72 l), the front of hemoglobin A_{Ic} reached the sintered disc K at the bottom, of the column. At this point, the chromatogram was stopped by closing outlet L and stopcock P. After the bolts had been removed from the flange A, the top half of the column which contained hemoglobin A_{II} (the main component), was lifted off and set aside on an aluminum plate. The dimensions of the column and the chromatographic conditions had been chosen so that only A_{Ic} would normally be present in the lower half of the column. Occasionally when a small amount of A_{II} had moved into the lower half, it could be readily scooped out. A_{Ic} and A_{II} usually were separated by several centimeters of almost white resin.

The lower half column B and its support system (D, F, G, H, I, J) were removed from the cold room, adapter S was attached and held in place with the flange, developer was added to the top (although passage of developer was still prevented at outlet L), and this section of the column was warmed by circulating water at 38° through the jacket. After the column had warmed to 38° (2 to 3 h), A_{Ic} was eluted at that temperature as rapidly as possible into a collecting vessel which was chilled in ice. Approximately 2 g of A_{Ic} were contained in a volume of about 2 l; the solution was concentrated at 2° through collodion bags (membrane filter, collodion bags type CB, porosity less than 5 mμ, size 8 ml, from the Membranfiltergesellschaft, Göttingen, Germany) to a final volume of 5 to 10 ml.

In the same manner, hemoglobin A_{II} was eluted from the top half of the column by the use of adapter T. Complete elution required elevation of the temperature to 50°.

Prior to the next chromatogram, all resin was removed and suspended in water. After the column had been repoured, it was equilibrated as described.

DISCUSSION

By means of the apparatus described, it has been possible to increase the scale of operations in the chromatographic isolation of hemoglobin by a factor of 20 over previous experiments. Although the saving in time and effort is appreciable, it is by no means a factor of 20.

Although this chromatographic tube has been used successfully for a specific purpose, the special features of demountability and transportability may make it valuable with modification for other applications.

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SUMMARY

The construction and operation of a demountable 10 × 100 cm chromatographic tube is described. In a specific application of this apparatus, a load of 50 g of adult human hemoglobin has been chromatographed for the isolation of 2 g of the minor hemoglobin component A_{Ic}.

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