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CONTINUOUS CHROMATOGRAPHY APPARATUS

II. OPERATION

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SUMMARY

Operating techniques for a continuous chromatography apparatus are given, as well as some general observations on the operation of such columns using molecular sieve gels.

INTRODUCTION

In the previous paper[1] we have described the construction of a continuous chromatography apparatus and this communication describes the operation of the column using molecular sieve gels. The techniques described, and the observations made will probably apply as well to other chromatographic media.

OPERATION OF THE COLUMN

Control of flow rate

Two factors are of importance to the operation of the column; first, that the flow rate be low enough to allow for full development and separation of the solute bands, and secondly, that the flow rate be the same throughout the column. With the standard size of Sephadex* gels (40–120 μ) the flow rate through the column was of the order of 3 to 4 l/h, which was too fast for resolution of the protein fractions. It was evident that some method of controlling the flow rate would have to be devised. In column chromatography the flow rate is under variable control and adjusted to allow sufficient time on the column for the fractions to separate, where resolution is a function of flow rate. Although reduction in flow rate was required, control thereof did not have to be variable, for resolution is also a function of the characteristics of the solvent-fixed phase system and the height of the column, both of which can be

* Mention of commercial names does not imply endorsement by the U.S. Department of Agriculture.

varied. The degree to which the vertical chromatographic separation is translated into a horizontal separation in the continuous system is a function of the speed of column rotation (to illustrate with the trivial case, if the column were not rotated, there would be no horizontal separation), so we incorporated a variable speed control into the column. Working with this limitation, a number of techniques for flow control were tried. Since some of the observations made are germane to the subject of the operation of the column, we will discuss them briefly.

We first tried to control flow by the size of the exit orifices, but a series of flow rate determinations using hypodermic needles of various sizes under simulated operating conditions led to the extrapolation that for the desired flow rate of 500 ml/h, through a column with 100 exit orifices, we should have to use a 37 or a 39 gauge needle. Over and above the impracticability of working with tubing of this size, especially with 100 tips required, it was felt that the tubing would be far too prone to plugging, usually with no hope of unplugging during operation of the column. For the same reason, that is, the fineness of the orifice required, no extensive trials were held of needle valve closures or other techniques since the orifice of the closure would have to be of the same or smaller dimension than the aforementioned tubing.

Another possibility investigated was the use of the filter paper support medium to control the flow rate. Filter papers of various makes and porosities were tried, with pore sizes ranging down to 0.25 μ. Regardless of initial flow rate, which in some cases was satisfactory, the column flow rate would constantly decrease and eventually stop altogether. At first this was thought to be due to deterioration of the support medium, but investigation with inert filter materials (Millipore's Solvinert and Polyvic; glass fiber filter paper) demonstrated this was not the cause. It was observed that debris collected on the filter paper and it thus appeared that the decreasing flow rate was a clogging problem. Obviously, care in prefiltering solvent and solution could reduce the problem, but *a priori* considerations make it doubtful that clogging could be eliminated. A total consideration would be required of the production of fine particles from gel breakdown, protein denaturation, the effect of exposure to atmosphere during manipulation, etc. Such a study did not appear fruitful to the author, and no further work was done.

What was needed was flow control without fine orifices, which meant either positive pumping, precluded in this instrument, or use of fixed phase material that would itself control the flow rate. In accord with the latter, Sephadex gels of the Superfine grade (10–40 μ) were tried, and found to give a satisfactory flow rate, about 1 l/h. As described later the protein bands came off the column within 1 h after application. Use of the Superfine gel also resulted in better resolution.

The gels were supported on the column by using a coarse-grade rayon filter paper, Union Carbide's Miracloth. No detectable change in flow rate occurs as long as large particles from protein denaturation or bacterial growth do not occur, and it is assumed that fine particles such as dust pass through the column. Since both the filter paper and the drip tips have relatively large orifices there is no danger of localized plugging resulting in uneven flow rates and wavering bands.

Uniform flow rates

Evenness of flow around the circumference of the column is necessary to

maintain a constant exit position of the several solute bands. Localized slowing of the flow rate results in longer times on the column for all of the solute bands in the area; the bands of solute are therefore transported further around the circumference and the exit positions of the solutes move in the direction of rotation. An even flow rate throughout the column has been found to be a function of the gel and the technique used to level the top surface of the fixed phase bed.

The gel

The gel used has to have the necessary characteristics, such as uniform particle size and shape, uniform exchange characteristics, uniform packing and compressibility characteristics, etc., which give uniform flow rates. The Sephadex gels have these characteristics developed to a sufficient extent to make the system practicable, although some improvements could be made. Since our experience to date has been only with the Sephadex gels, we can make no comments concerning other products. The gels were used directly as they come packaged. The dry material was stirred into the appropriate solvent, allowed to stand for a day, and poured on the column; the exact technique is described later. The gels used are compressible, however, which is one of the reasons we did not provide for shutting off the column. Sephadex gels, particularly the higher number gels (larger pore size) compress during flow, and the height of the column is considerably shortened. When the flow is shut off the height increases. In a large cylindrical column, this probably would not produce localized packing, but in the narrow confines of the cylindrical annulus used for the continuous apparatus, it was speculated that localized packing could occur. It therefore appears *a priori* that column shut-off would be inadvisable and the inability to do so has not been any inconvenience.

The gels also exhibited flow variation with temperature, but enclosing the column eliminated that problem.

Assuming then that the gel, or other fixed phase material, is suitable for continuous chromatography, the technique of preparing the column becomes the deciding factor in obtaining even flow and resolution of solute fractions.

Preparing the gel bed

The gel was suspended in the solvent and poured into the loading reservoir, which in our apparatus consisted of the top half of a 4-l polyethylene jug, inverted, and stoppered with a one-hole stopper, with a length of rubber tubing leading down to the column. The loading reservoir is on top of the case and the rubber tubing is led through a hole in the top of the case. The column is rotated at a speed of 1.33 r.p.m. and the gel allowed to drain on to the column. When all of the gel is on the column, the reservoir is rinsed, and the solvent feed system turned on to maintain the solvent head.

After the gel has settled, it is then necessary to level the top surface of the bed, which is done with the leveling plow previously described. The plow is mounted in the reed movement, and a micro-valve connects the needle to a solvent supply in the loading reservoir. The solvent flow is adjusted with the needle well above the gel bed. The plow is then lowered to just above the bed and clamped securely in the reed movement. The column is rotated with the rotator at 1.33 r.p.m., the solvent flow turned on gently, and the plow lowered into the bed surface (Fig. 1). The blade of

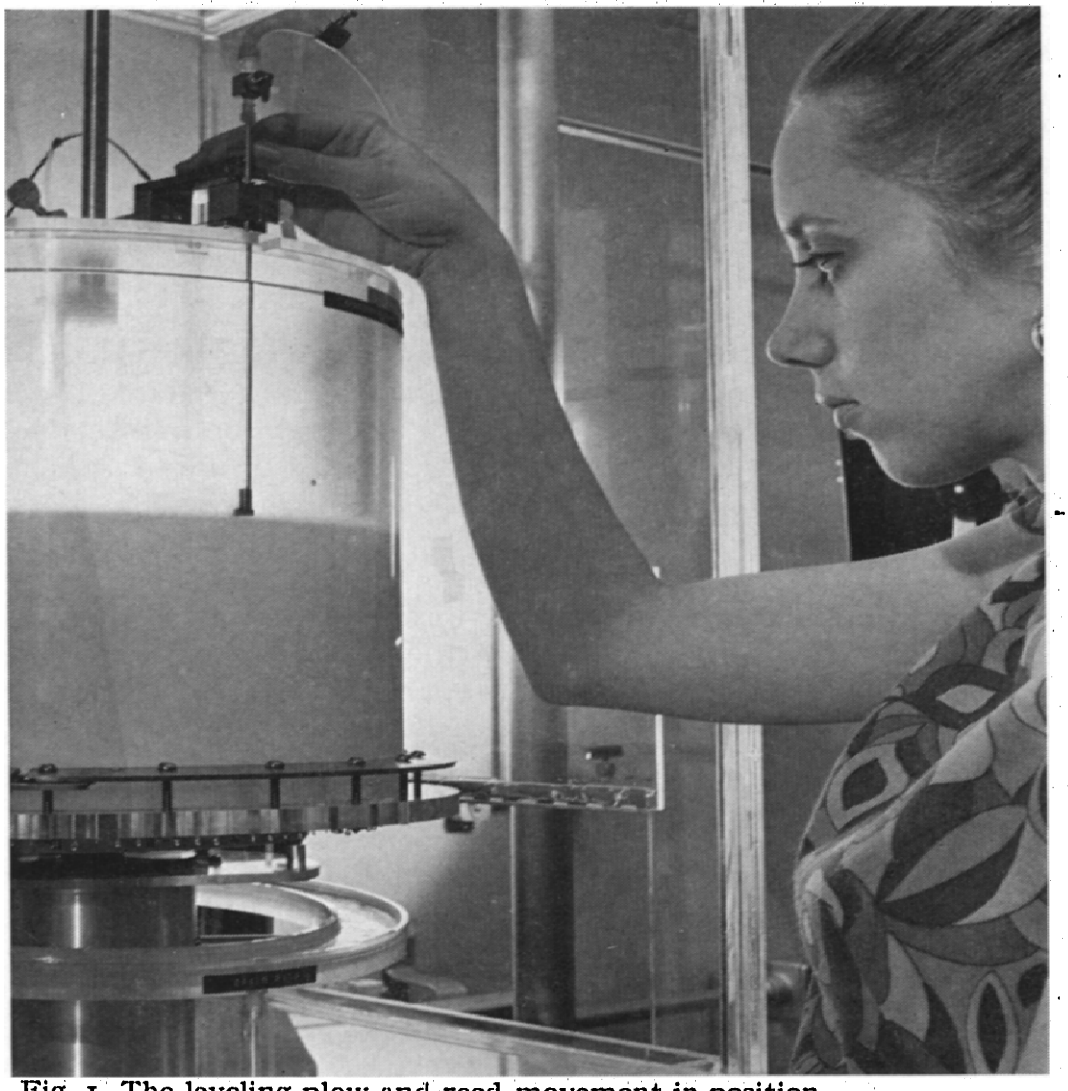

Fig. 1. The leveling plow and reed movement in position.

the plow lifts the top surface of the fixed phase bed and the jet of solvent disperses it (Fig. 2). To remedy highly uneven packing of the bed it is sometimes desirable to turn on the solvent with some force and disperse the fixed phase with the jet. After the top surface has been dispersed, the flow of solvent is shut off and the column allowed to rotate. As the fixed phase resettles, the plow is lifted by degrees until the fixed phase no longer balls up in front of the plow. At this point the bed should be quite level, having neither high nor low points, and it should not slope either to the inside or to the outside of the column. The plow is then withdrawn. This phase of the operation is most critical. Care should be exercised not to allow the plow to drop into the surface of the gel, for if it does a compressed area results and a distortion in the bands develops which will not disappear. It is largely a matter of technique, which requires some practice.

Sometimes the column becomes clogged with debris, denatured protein and/or bacterial growth. When this happens, the column bed material disperses in strings or clumps in the jet of buffer from the plow. The condition is alleviated usually, and normal flow rates restored, by aspirating the top layer of material. The needle is

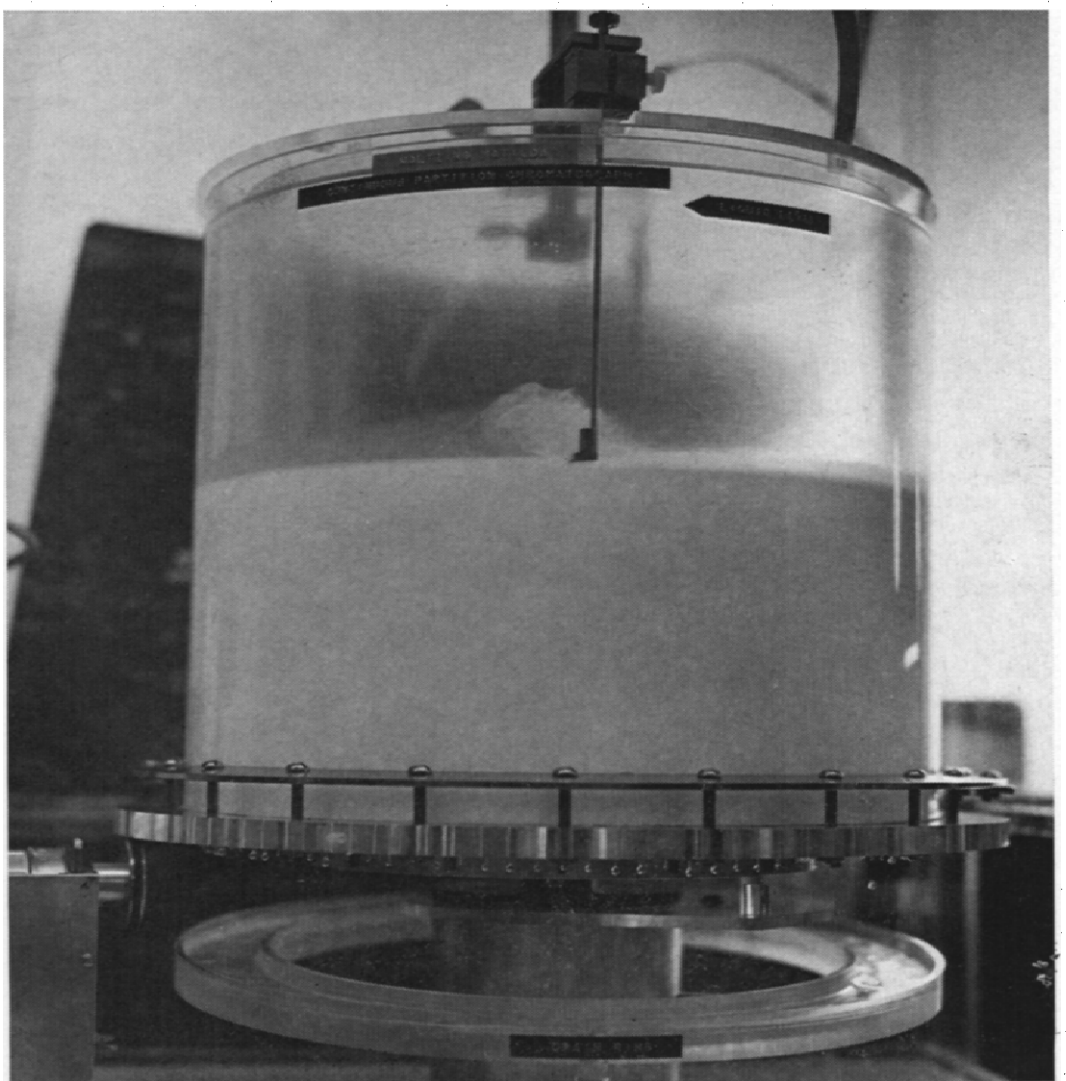

Fig. 2. The dispersing of the fixed phase by the jet of solvent from the leveling plow. Note the high-speed column rotator in position at lower left.

connected to a water aspirator, and with solvent flowing into the plow orifice, the plow is lowered into the fixed phase bed while the column is rotated in the normal direction. The top layer is sucked off until the bed material flowing into the plow orifice is no longer lumpy, but is finely dispersed. For the operations carried out in this laboratory usually only the top millimeter or two was removed. If the column became too contaminated we removed the fixed bed material (Sephadex) and boiled it for an hour or two, skimming the top occasionally. This kept the bacterial population down, removed denatured protein, decolorized the material and restored normal flow rates.

Operating technique

Once the bed has been leveled, the reed movement is placed over the site where solution is to be applied. The applicator needle and tubing are filled with solution and the needle placed in the reed movement with the long axis of the flattened tip on a radius. The needle is lowered to within 2–3 mm of the surface of the fixed phase,

the column turntable started, and the solution pump turned on. After the solution has layered on the top of the bed and equilibrium conditions have been established, the input flow rate is adjusted to the desired rate and the needle lowered so that there is a very slight vertical flow of solution above the solution already layered on the fixed phase bed. If the needle is too low it will spread the solution as the column rotates, and if it is too high too much mixing occurs. If the solution is visible to the eye, the manner in which it layers on the column can be observed. If it layers evenly across the width of the column, the bed surface is satisfactory. If, however, the solution runs to the front or the back, collects in a trough in the middle, or runs in either direction, the top of the bed is not level and for best separations the operation should be stopped and the bed releveled. Finally, once the desired input conditions have been satisfied, the solution input flow rate and the speed of column rotation are adjusted to give the desired separation of emerging bands.

Collecting the fractions

The collection device has been described previously[1] and was operated in either of two ways. For collection of individual fractions to determine the operating characteristics, all of the collection cones were filled with diverting tubes which terminated over 25 tubes in a rack curved to fit just outside the drain ring. For collection of all 100 possible fractions the collecting device was moved to successive quadrants during operation of the column. For collecting just one solute fraction, the diverting tubes were removed from all but as many collecting cones as would collect the entire fraction. The section with the remaining diverting tubes would be positioned under the site of emergence of the desired band, and a beaker placed under the outer ends of the diverting tubes.

This completes the description of the operating technique evolved in this laboratory for the operation of the continuous chromatography apparatus. The subsequent section[2] will describe two separations achieved with the apparatus, one for hemoglobin/myoglobin and one for milk proteins/salt–lactose.

ACKNOWLEDGEMENT

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REFERENCES

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