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THE AUTOGRAD

AN AUTOMATED SIMPLIFIED ELUENT GRADIENT-GENERATING SYSTEM FOR USE IN LIQUID CHROMATOGRAPHY*

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SUMMARY

An automated two-chambered gradient-generating system has been developed. This system, called the Autograd, can be mathematically designed to duplicate simply and reproducibly the performance of complex gradient generators. In addition, it provides automated regeneration of the chromatographic column in preparation for the next analysis, an important feature for analyses of a continuing, repetitive nature. Typical chromatographic results are presented to illustrate the usefulness of such a device for routine chromatographic operation.

INTRODUCTION

The use of gradient elution (the process by which the properties of the eluent are changed with time or elution volume) is well established in all phases of liquid chromatography[1]. Many devices have been developed to produce the necessary concentration gradients in the eluent in order to effect a desired separation. These devices range in complexity from simple two-chambered systems[2,3] to complicated machines involving photoelectric curve followers and variable speed pumps[4].

One of the most popular of these devices has been the nine-chambered Varigrad[5], which exhibits great versatility with respect to the large variety of gradients that can be produced. However, the flexibility of complex gradient generators is often not necessary for routine analyses. Also, the excessive operator time required and the threat of various mechanical failures are distinct disadvantages for gradient generators of this type.

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For routine operation, a simple gradient-generating system is preferred. Simplicity in this case refers not only to the mechanics of the device itself but also to minimal operator time for mixing the various components of the eluents and preparing the generating device for the next analysis. In addition, the system should contain provisions for column regeneration and be amenable to complete automation. The purpose of this communication is to discuss the design and operation of an automated, two-chambered system that requires minimal operator attention. The basic characteristics of this device, called the Autograd, have been briefly described previously[6].

MATERIALS AND METHODS

Design

The Autograd, shown in Figs. 1 and 2, utilizes two chambers to form the eluent gradient. One chamber is filled with a concentrated eluent, the other with a dilute eluent. The two eluents flow simultaneously from their respective chambers into a common mixing tee, and then the combined stream is introduced onto the chromatographic column. Since the cross-sectional area at the top of the dilute-eluent chamber is large compared with the cross-sectional area at the top of the concentrated-eluent chamber, the ratio of dilute to concentrated eluent in the mixing chamber at the beginning of the analysis is large and the combined eluent that passes to the column is dilute. However, the cross-sectional area at the bottom of the dilute-eluent chamber is small compared with the cross-sectional area at the bottom of the concentrated-eluent chamber, and the ratio of dilute to concentrated eluent in the mixing chamber at the completion of the analysis is also small. Thus the combined eluent that is admitted to the column toward the end of a run is concentrated. The shape of the two wedge-like containers determines the eluent gradient profile throughout the analysis.

Discussion of the actual design of the Autograd necessitates a few definitions and explanations. In Fig. 1, between points 1 and 2, the thickness or length (L) is the same for each chamber and constant with height (H). The side dimension or width (W) varies and is a function of the height. When the chambers are viewed from the side, one edge is seen to be perpendicular to the base, whereas portions of the other edge are angled with respect to the base. The angled edges of each chamber are complementary. (Note that the dimensions of interest are inside dimensions.)

Tailoring the Autograd to duplicate the performance of a more complex gradient-generating device is not complicated, provided the shape of the desired gradient (eluent properties *versus* elution volume) is known and is not too complex. Since an exact mathematical analysis of the gradient for the nine-chambered Varigrad has been published[7,8] one can construct a theoretical curve of the Varigrad output for any combination of buffers desired. Alternatively, the concentration profile can be obtained by monitoring directly the gradient from an existing gradient-generating device by means of a suitable detector (*e.g.*, a conductivity meter) for the ionic strength of the eluent. The height, combined width, and length are adjusted to give the appropriate total volume of eluent to be passed through the column per analysis and also to give the chambers a reasonable shape from the standpoint of operation and fabrication.

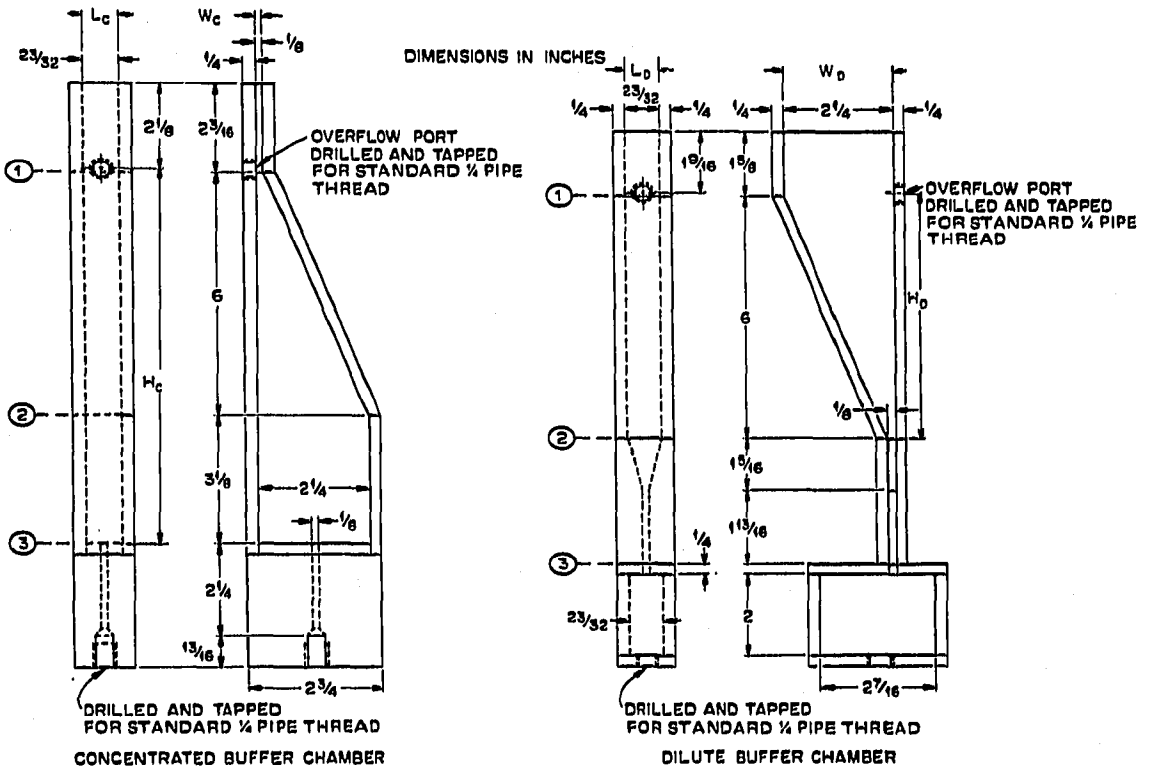

Fig. 1. The Autograd for the Carbohydrate Analyzer.

Since the ratio of the cross-sectional areas at the top and the bottom of the dilute and concentrated chambers determines the initial and final eluent concentration of the gradient, respectively, these areas are normally determined first, under the constraint that the width of any portion of either chamber cannot be less than about $^1/_8$ in. If the width is less than this value, two problems arise: simplicity in fabrication of the system is lost, and surface tension in this narrow region becomes significant and tends to disrupt the expected performance of the device.

In order to calculate the ratio of cross-sectional areas of dilute- and concentrated-eluent chambers at the top and bottom of each of the two chambers, we must first derive a relationship between the cross-sectional areas, the concentrations of the eluents in the chambers, and the concentration of mixed eluent issuing from the mixing tee. When a certain volume V_1 of concentration C_1 is mixed with another volume V_2 of concentration C_2, the average concentration $\bar{C}$ that results is given by the expression

$$C_1V_1 + C_2V_2 = \bar{C}\,(V_1 + V_2) \qquad (1)$$

i.e., the concentration of the mixture is just the average concentration, on a volumetric basis, of the two starting solutions. Eqn. 1 can be rewritten as

$$\frac{C_1V_1 + C_2V_2}{V_1 + V_2} = \bar{C} \qquad (2)$$

This equation also describes the mean concentration of the effluent from a two-chambered gradient-generating device which feeds the solution in each chamber into a common mixing tee, since two solutions of different concentrations are being mixed to form the eluent for the chromatographic analysis. Thus, for a differential drop dx_1 of fluid level in chamber 1 and dx_2 of fluid level in chamber 2, we have

$$\frac{C_1A_1\,dx_1 + C_2A_2\,dx_2}{A_1\,dx_1 + A_2\,dx_2} = C \tag{3}$$

where A is the cross-sectional area of the chamber at the eluent level in that chamber. Since the chambers are connected, the two solutions are in hydrostatic equilibrium. Therefore,

$$\zeta_1x_1 = \zeta_2x_2 \tag{4}$$

where ζ_1 = density of fluid in chamber 1;
ζ_2 = density of fluid in chamber 2;
x_1 = height of fluid in chamber 1;
x_2 = height of fluid in chamber 2.

Thus,

$$\zeta_1\,dx_1 = \zeta_2\,dx_2 \tag{5}$$

and, substituting eqn. 5 in eqn. 3, we have

$$\frac{C_1A_1\zeta_2 + C_2A_2\zeta_1}{A_1\zeta_2 + A_2\zeta_1} = C \tag{6}$$

This equation relates the cross-sectional areas of the two chambers to the concentration of eluent leaving the mixing tee, in terms of the concentrations and densities of the solutions in the two chambers.

For example, assume that the molar concentrations of the two starting eluents are 0.01 and 1.0, respectively, and an initial effluent concentration of 0.02 M is desired. The equation that describes the cross-sectional areas at the top of the dilute- and concentrated-eluent chambers (point 1, Fig. 1), assuming $\zeta_1 = \zeta_2$, would be

$$\frac{0.01\,A_{D_1} + 1.0\,A_{C_1}}{A_{D_1} + A_{C_1}} = 0.02 \tag{7}$$

where A_{D_1} = cross-sectional area at the top of the dilute-eluent chamber (point 1, Fig. 1);
A_{C_1} = cross-sectional area at the top of the concentrated-eluent chamber (point 1, Fig. 1).

Since the cross-sectional area is equal to the product of the length of the chamber and the width of the chamber, eqn. 7 can be rewritten as

$$\frac{0.01\,W_{D_1}L_{D_1} + 1.0\,W_{C_1}L_{C_1}}{W_{D_1}L_{D_1} + W_{C_1}L_{C_1}} = 0.02 \tag{8}$$

where W_{D_1} = width of the dilute-eluent chamber at the top (point 1, Fig. 1);
W_{C_1} = width of the concentrated-eluent chamber at the top (point 1, Fig. 1);

L_{D1} = length of the dilute-eluent chamber at the top (point 1, Fig. 1);
L_{C1} = length of the concentrated-eluent chamber at the top (point 1, Fig. 1).
For the system shown in Fig. 1, we have

$$L_{D1} = L_{C1}$$

hence, in this case, eqn. 8 reduces to

$$\frac{0.01\,W_{D1} + 1.0\,W_{C1}}{W_{D1} + W_{C1}} = 0.02 \tag{9}$$

Since we have one equation and both W_{D1} and W_{C1} are unknown, we can only solve for the ratio of the widths. However, if the sum of the two widths is arbitrarily set equal to some number, then we can solve for each individual width. In designing the chambers, this procedure is followed. For example, if we choose

$$W_{D1} + W_{C1} = 5 \text{ in.} \tag{10}$$

we can solve eqns. 9 and 10 simultaneously to find

$$W_{C1} = 0.0505 \text{ in.} \tag{11}$$

This value for W_{C1} is considerably less than our lower limit of $^1/_8$ in. Thus, for this example, $W_{C1} + W_{D1}$ should be increased. Under certain conditions, however, the

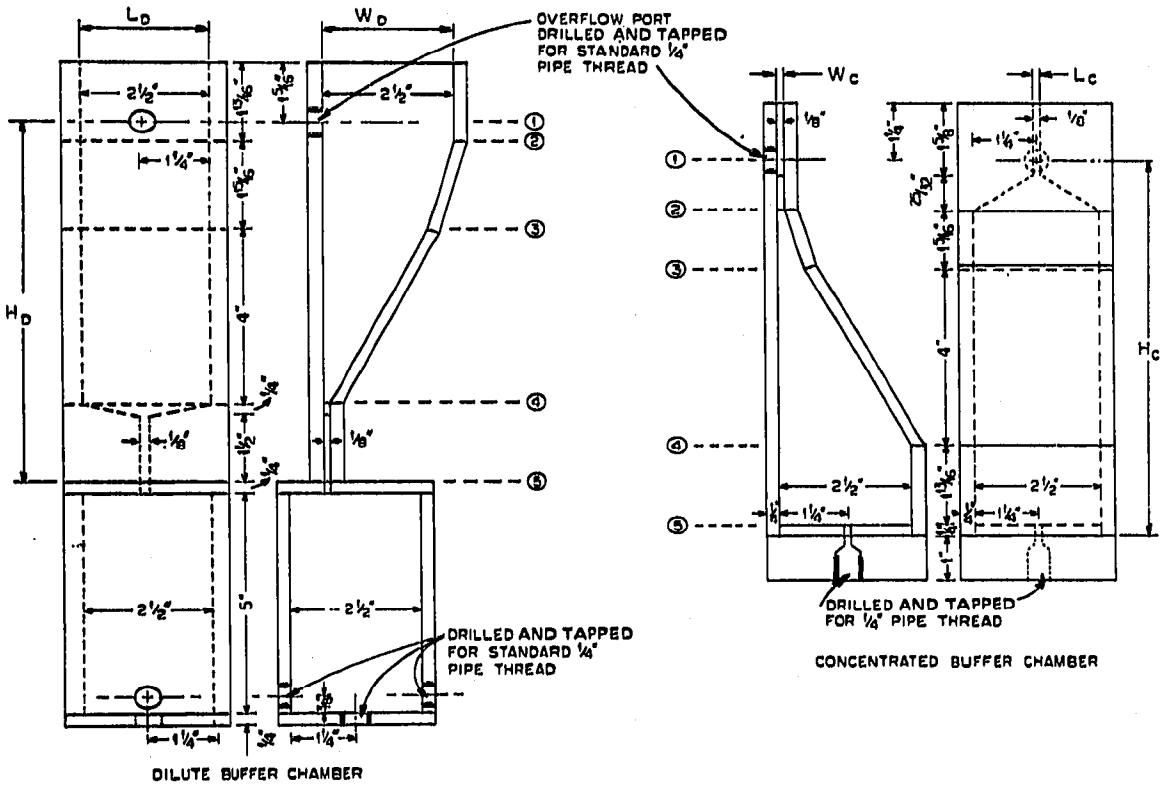

Fig. 2. The Autograd for the UV Analyzer.

difference in molarities of the two starting buffers may be so great that the above constraint leads to a value for the combined width ($W_{D_1} + W_{C_1}$) which is large enough to be both impractical and unwieldy. In such a case, a useful scheme is to set W_{C_1} equal to $^1/_8$ in. and then begin decreasing L_{C_1} until the proper ratio between the two areas A_{C_1} and A_{D_1} in eqn. 6 is achieved (constraint: $L_{C_1} \geq {}^1/_8$ in.). Since L_{D_1} and W_{C_1} are chosen, we must select a value for W_{D_1} in order to calculate L_{C_1}. This approach is illustrated in Fig. 2, which depicts the Autograd for our recently developed UV analyzer[9-11]. This system was specifically designed for a 0.45 × 150 cm column. As shown in Fig. 2, both the length and the width at the top of the concentrated-eluent chamber in this system are constricted; in contrast, the width of the concentrated-eluent chamber in Fig. 1 varies between points 1 and 2, while the length remains the same.

Once the cross-sectional areas at the top and bottom of the two chambers have been determined, the shape and height of the chambers that will give the desired gradient profile remain to be calculated. The actual gradient profile of the gradient generator that is to be duplicated by the Autograd is needed for this final step.

For example, Fig. 1 shows the Autograd that was designed and built for our recently developed Carbohydrate Analyzer[12]. This analyzer originally employed a nine-chambered Varigrad to produce the buffer gradient. Fig. 3 shows the theoretical curve for the buffer gradient generated by the Varigrad for the Carbohydrate Analyzer. For design purposes, this smooth curve, which was obtained by means of the computer program previously described[8], must be simulated by as few straight lines as possible. The resulting broken curve, also shown in Fig. 3, serves as the basis for the design of the Autograd. The Autograd is constructed in such a manner that the eluent gradient it generates duplicates the broken curve. The small mixing chamber serves not only to mix the two streams but also to smooth out the sharp breaks in the gradient curve, thereby reducing the disparity between the performance of the two systems.

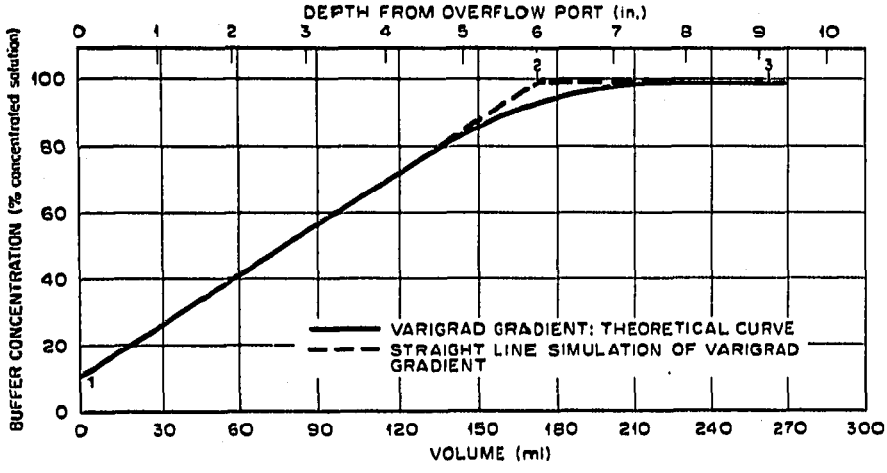

Fig. 3. Straight line simulation of Varigrad gradient for Carbohydrate Analyzer.

The next step is determination of the width of each chamber at the chamber heights corresponding to point 2 in Fig. 1 and points 2–4 in Fig. 2. These widths are determined by calculating, using eqn. 6, what ratio of the combined widths of the two chambers would give the desired buffer concentration at point 2 in Fig. 3, based on the known concentration of buffer in each chamber. Once the widths of the chambers have been calculated for the top (point 1), bottom (point 3), and each break in the gradient curve (point 2, Fig. 3, in the present example), the heights of each section can be calculated. From Fig. 1, the volume of the dilute chamber between sections 1 and 2 is:

$$V_{D(1-2)} = (A_{D1} + A_{D2})\, H_{D(1-2)}/2 \qquad (12)$$

where $H_{D(1-2)}$ is the height between sections 1 and 2 of the dilute-eluent chamber. Likewise, the volume of the concentrated-eluent chamber between sections 1 and 2 is:

$$V_{C(1-2)} = (A_{C1} + A_{C2})\, H_{C(1-2)}/2 \qquad (13)$$

where $H_{C(1-2)}$ is the height between sections 1 and 2 of the concentrated-eluent chamber. The combined volume of the two chambers between sections 1 and 2 must equal the elution volume corresponding to the desired eluent concentration at section 2, which is found from Fig. 3 to be 170 ml. Thus,

$$(A_{D1} + A_{D2})\, H_{D(1-2)}/2 + (A_{C1} + A_{C2})\, H_{C(1-2)}/2 = 170 \qquad (14)$$

Also, from eqn. 5:

$$\zeta_D H_{D(1-2)} = \zeta_C H_{C(1-2)} \qquad (15)$$

Since the areas in eqn. 14 have already been determined, the values of the heights of the two chambers between sections 1 and 2 can be determined from eqns. 14 and 15. The same procedure is followed to determine the heights of the two chambers between sections 2 and 3.

Once the total height of each chamber has been determined, the actual plans for the devices can be drawn up, as illustrated in Figs. 1 and 2. The individual chambers may be constructed of Lucite or some similar material.

Operation

When the Autograd is installed in the chromatographic analysis system, the two chambers must be balanced since the buffers in the two chambers are to be in hydrostatic equilibrium during the analysis. In general, the densities of the two eluents will not be the same; therefore, the two liquid levels will be different when the liquids are in hydrostatic equilibrium. The difference in heights of the overflow ports in each chamber above the mixing vessel must be set accordingly. Normally, shut-off valves will be included in the line connecting the outlets of the chambers to the mixing tee. The installed device is shown in Fig. 4. At the start of a chromatographic analysis, the chambers are filled until they overflow at a present level and require no further attention until the beginning of the next analysis. Only two buffers are required, and operator maintenance time is minimized. Since each chamber feeds into a small, magnetically stirred, common mixing chamber, it needs only to be refilled after a run is complete; no rinsing is required.

The reservoir attached to the bottom of the dilute chamber (*cf.* Figs. 1 and 2)

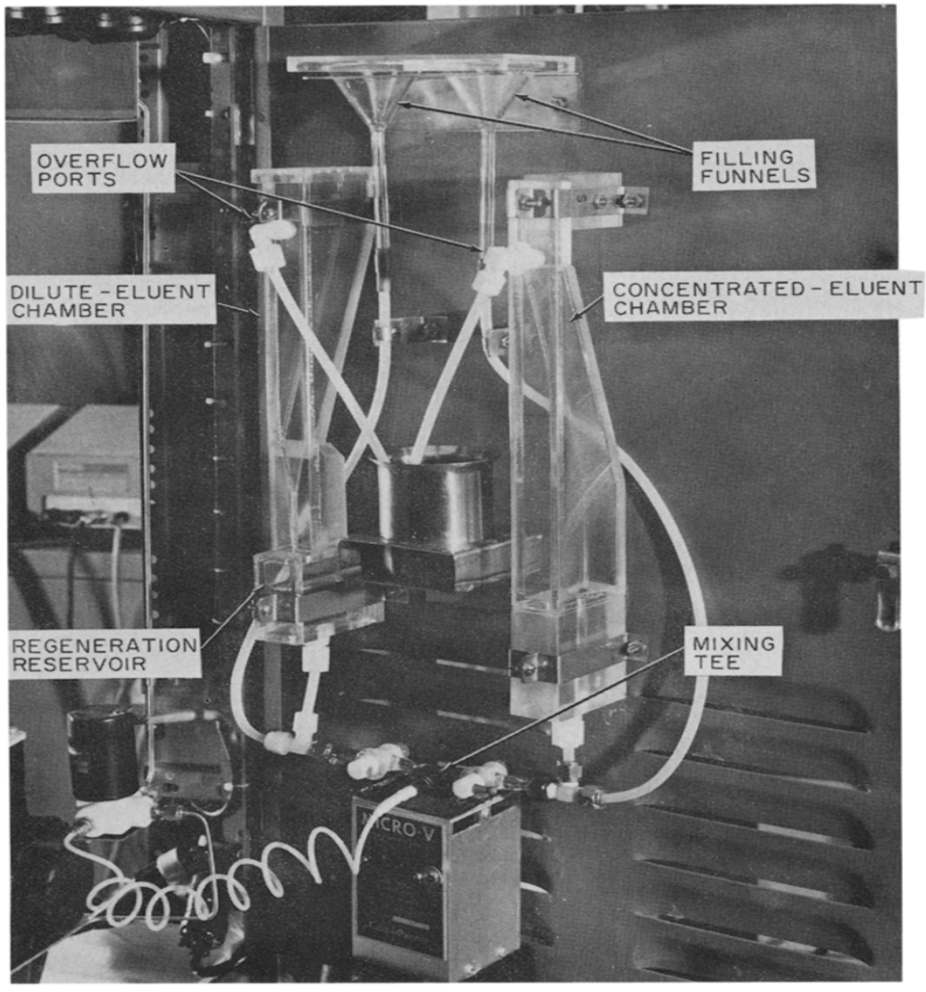

Fig. 4. Autograd installed in the Carbohydrate Analyzer.

contains a volume of buffer sufficient to regenerate the column between runs. During the regeneration phase, two modes of operation are possible. In one mode, the line leading from the concentrated buffer chamber to the mixing chamber is closed by means of a timer-activated solenoid valve. The column is then regenerated by the buffer in the dilute chamber reservoir. In the second mode, the two chambers of the Autograd are designed so that the concentration of the mixture of buffers from the two chambers during the regeneration step is the same as that produced at the beginning of the analysis. The actual chromatographic results of any particular analysis will determine which approach to follow, although the latter is preferred from the standpoint of mechanical simplicity. In either case, the small mixing chamber is rinsed out in-line during the column regeneration step.

RESULTS AND DISCUSSION

Recently, a small, automated, high-resolution analyzer for determination of carbohydrates in body fluids (*i.e.*, the Carbohydrate Analyzer) was developed at ORNL[12]. This device was designed to separate borate complexes of carbohydrates on a column of strong anion-exchange resin, using a sodium borate–boric acid buffer gradient. The eluent gradient was initially generated by a nine-chambered Varigrad, employing four different solutions. The Varigrad has since been replaced by the Autograd, shown in Fig. 1.

The Autograd was designed to duplicate the performance of the Varigrad in the Carbohydrate Analyzer, by employing a concentrated borate buffer at pH 8.6 (0.147 mole of sodium borate and 0.283 mole of boric acid per liter) in the concentrated-eluent chamber and a 5% solution in the dilute-eluent chamber. The concentration of the borate buffer produced by the Varigrad ranged from 10% at the start of the analysis to 100% at completion. To illustrate the similarity in the concentration gradients between the nine-chambered Varigrad and the Autograd, the mixing chamber of the Autograd was filled with the 5% buffer and connected to a Milton Roy minipump, which removed buffer from the system at the constant rate of 106 ml/h. The buffer concentration in the effluent leaving the mixing chamber was monitored by a conductivity meter. A nine-chambered Varigrad, prepared for a normal carbohydrate analysis (see ref. 12), was then connected to the pump, and the concentration gradient of this device was monitored in a similar fashion. Fig. 5 shows the results of these tests. Although the slopes of both gradient curves are equal, signifying that the Autograd had been designed properly, the curve resulting from the two-chambered device was displaced slightly to the right because 5% buffer was present in the mixing chamber at the beginning of the run.

When the Autograd was installed in the Carbohydrate Analyzer, the more dilute buffer (5%) at the outset of the chromatographic analysis caused a noticeable

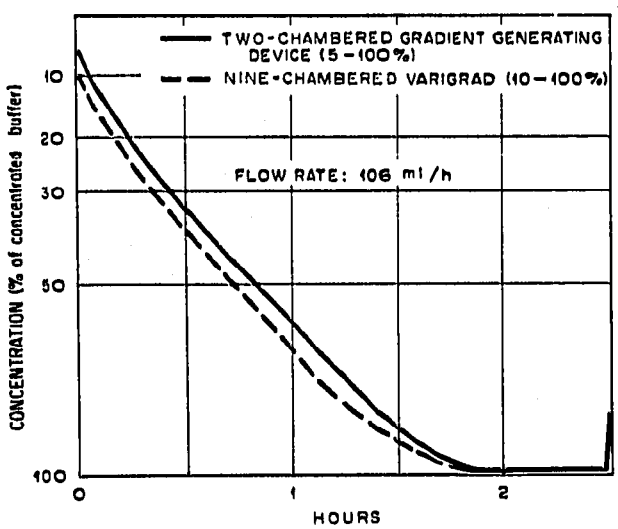

Fig. 5. Comparison of concentration gradients of Varigrad and Autograd.

increase in band widths of the early-eluting compounds. Therefore, the concentration of the buffer in the dilute chamber was increased to 10%, which resulted in chromatograms of sugar standards essentially identical to those obtained when the Varigrad was employed (Fig. 6).

Although the chromatograms show that, in each case, essentially the same resolution was obtained with respect to relative elution positions of the peaks, there are noticeable differences in the elution times of the peaks. These differences are due to a higher flow-rate used when the Autograd was being tested.

CONCLUSION

The device used for generating the gradient in liquid chromatography can often be greatly simplified, not only with regard to the mechanics of the device itself but also from the standpoint of operator time necessary to maintain the device, by replacing it with an automated, two-chambered gradient-generating system, referred to as the Autograd, which requires minimal operator attention. The Autograd has successfully replaced the nine-chambered Varigrad, which requires four separate buffers and a separate column regeneration system, in the recently developed ORNL Carbohydrate Analyzer.

The Autograd requires only two buffers, and its chambers have no moving parts. The chambers are filled until they overflow at a preset level at the beginning of a run and require no further attention until the beginning of the subsequent run. Thus the Autograd is completely automated, and operator maintenance time is minimized. Since this system can be designed to closely duplicate the performance of more complex gradient generators, it presents a distinct improvement for routine analyses, allowing the operator more time for duties of a less routine nature.

SYMBOLS

A_{C_1} = cross-sectional area of concentrated-eluent chamber at section 1
A_{D_2} = cross-sectional area of dilute-eluent chamber at section 2
C_1 = concentration of the dilute eluent
C_2 = concentration of the concentrated eluent
$\bar{C}$ = average concentration of mixed eluents
$H_{D(1-2)}$ = height of dilute-eluent chamber between sections 1 and 2
$H_{C(1-2)}$ = height of concentrated-eluent chamber between sections 1 and 2
L_{C_1} = length of concentrated-eluent chamber at section 1
L_{D_2} = length of dilute-eluent chamber at section 2
$V_{D(1-2)}$ = volume of the dilute-eluent chamber between sections 1 and 2
$V_{C(1-2)}$ = volume of the concentrated-eluent chamber between sections 1 and 2
W_{D_1} = width of dilute-eluent chamber at section 1
W_{C_2} = width of concentrated eluent chamber at section 2
x_1 = height of diluteeluent above mixing tee
x_2 = height of concentrated eluent above mixing tee
dx_1 = differential drop in height of dilute eluent
dx_2 = differential drop in height of concentrated eluent
ζ_1 = density of dilute eluent
ζ_2 = density of concentrated eluent

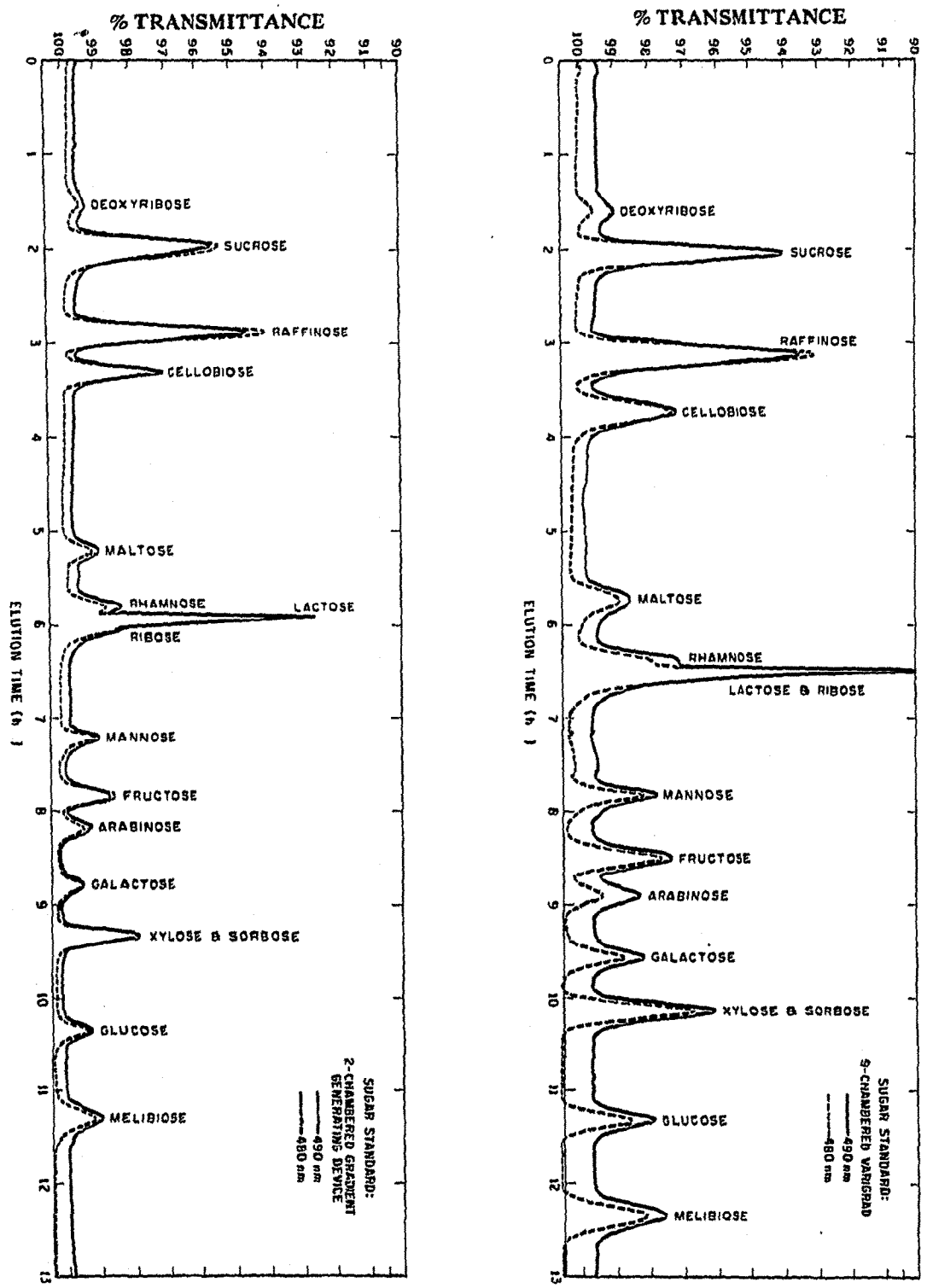

Fig. 6. Comparison of standard sugar chromatograms obtained by using Varigrad and Autograd.

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