H_3BO_3 at pH 8.5, and 0.150M H_3BO_3 at pH 9.0, respectively. While all of the peaks are not completely resolved, an increase in column length would most likely improve resolution greatly. Initial chromatograms of the same mixture on a column 750 mm long showed celloheptaose and cellohexaose barely distinguishable as separate peaks. Using the same resin in a 1000-mm column gave the chromatogram in Figure 9. All of the cellodextrins except celloheptaose were easily soluble in 0.10M boric acid adjusted to pH 7.0 with sodium hydroxide. Celloheptaose entered solution completely after several hours stirring at about 40° C. Higher homologs of glucose have not been used in this system, but it is doubtful if any beyond the nonaose could be dissolved without increasing the alkalinity of the solvent. This would present the possibility of splitting, particularly at the column temperatures used.

The peaks in the chromatogram of the pulp hydrolyzate in Figure 10 represent the following polymer contents of the pulp, expressed as per cent by weight of dry pulp: mannan 6.9, araban 0.7, galactan 0.9, xylan 5.6, and glucan 69.1. While the first 2 hours of the chromatogram are not shown in Figure 10, three very small, but sharp and narrow peaks occurred, two of which corresponded to the furfural and hydroxymethyl furfural positions, and the third to the position normally occupied by rhamnose.

CONCLUSIONS

Ion-exchange chromatography of borate–carbohydrate complex anions on columns of strong-base anion-exchange resins offers a relatively rapid method of quantitative analysis of sugar mixtures, particularly when column effluents are monitored for sugars by an automatic analyzer. There are no derivatives to prepare, and a properly sized column can be used simultaneously for analytical and preparative purposes. The same method is applicable to other areas of carbohydrate chemistry, having been shown to have the ability to rapidly resolve mixtures of oligosaccharides and hemicelluloses in solution. The amounts of individual saccharides measurable can vary in a wide range from less than a microgram, using a 3-mm diameter column, to several decigrams on a 1-inch column.

ACKNOWLEDGMENT

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Fast Liquid Chromatography: An Investigation of Operating Parameters and the Separation of Nucleotides on Pellicular Ion Exchangers

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A liquid chromatographic system featuring high inlet pressures and a sensitive UV detector was designed for fast analysis of nonvolatile organic compounds. To establish optimal operating conditions, band dispersion in the mobile phase was studied by using capillary tubes as well as small bore columns packed with glass beads. Although peak broadening in open tubes was less than predicted by theory, the use of packed columns was more promising for fast separations. Novel pellicular column materials were prepared by coating glass beads with ion exchange resin and other solid phases. Rapid separation of nanomole quantities of ribonucleoside mono-, di-, and triphosphates was achieved by using a pellicular basic ion exchanger and gradient elution with a phosphate buffer. As shown by cellular extract analyses, the stability and efficiency provided by such column materials makes it possible to achieve fast separation of complex mixtures by a liquid chromatographic technique similar in speed, resolution, and quantitative range to gas chromatography.

A TECHNIQUE for the separation of nonvolatile substances which encompasses the speed, efficiency, sensitivity, and versatility of gas chromatography has yet to be devised. The present study was undertaken in an effort to investigate the feasibility of developing a liquid chromatographic system which could approach this goal. The method which evolved was then utilized for the analysis of the nucleoside phosphoric acids.

The time required for the separation of a solute pair is conveniently expressed by the retention time of the second peak, t, as

$$t = NH/u_b \quad (1)$$

where N is the number of plates required for the particular separation, H is the plate height, and u_b is the band velocity. Although H is the basic measure of the peak broadening, H/u_b, the time per plate, is more important if the speed of separation is of concern, as it is directly related to the separation time as shown in Equation 1. Since $u_b = u/(1 + k)$, Equation 1 can be written

$$H/u_b = t/n = H(1 + k)/u \quad (2)$$

where k is the column capacity ratio for the solute, and u the fluid velocity.

The plate height depends on the flow velocity (*1*, *2*) and, as Giddings (*3*) pointed out, if the molecular diffusion is the

(1) J. J. van Deemter, F. J. Zuiderweg, and A. Klinkenberg, *Chem. Eng. Sci.*, **5**, 271 (1956).
(2) M. J. E. Golay, ANAL. CHEM., **29**, 928 (1957).
(3) J. C. Giddings, *Ibid.*, **35**, 2215 (1963).

sole mass transfer mechanism, analogous operating conditions in gas and liquid chromatography would require flow velocities 10^5 times lower in liquid than in gas chromatography because diffusion coefficients are of the order of 10^{-6} cm^2 second^{-1} in liquids and 10^{-1} cm^2 second^{-1} in gases: consequently the time of analysis would be increased by the same factor. Obviously, high speed liquid chromatography cannot occur under those analogous conditions.

One can express the plate height (neglecting longitudinal diffusion) as the sum of three terms

$$H = f(H_m/u) + C_{m2}u + C_s u \quad (3)$$

$$H/u = H_m/u + C_{m2} + C_s \quad (4)$$

The first term, H_m, represents the plate height contribution due to peak dispersion in the mobile phase streaming through the column; the dependence of this term on flow velocity is not completely understood. The second term, $C_{m2}u$, stems from the mass transfer resistance by the stagnant mobile phase in the intraparticle pores of the packing. The third term, $C_s u$, is related to nonequilibrium due to the stationary phase. The three terms of Equation 4 should be minimized in order to obtain an increase in speed of analysis.

One conceivable approach is to reduce the particle size in packed columns. With porous granules C_{m2}, and with conventional ion exchange resins, C_{m2} and C_s, decrease with the square of the particle diameter. Thus, extremely fine silica particles were used for fast separation in liquid–solid chromatography (*4*), and significant acceleration of the analysis of amino acids and peptides by ion exchange chromatography (*5*, *6*) has been achieved by using very small uniform spherical resins.

Another approach involves the use of open tubular columns or columns packed with a fluid-impermeable support where $C_{m2} = 0$, and where C_s can be small if the depth of the proper stationary phase is small. Under these conditions the velocity-dependent first term, H_m/u, in Equation 4, which arises from the nonuniform flow pattern, becomes very important.

For laminar flow in straight open tubes according to Taylor (*7*),

$$H_m/u = \frac{d^2}{24\ D_M} \quad (5)$$

where d is the tube diameter and D_M is the diffusion coefficient of the solute in the mobile phase. This term becomes very high for a liquid mobile phase as a consequence of low diffusivity in liquids, and the use of open tubular columns does not appear to be favorable under laminar flow conditions in liquid chromatography.

Pretorius and Smuts (*8*) have proposed the operation of open tubular columns with turbulent flow which is characterized by very low H/u values. In gas chromatography, this effect of turbulence has been proved experimentally (*9*) and an abrupt drop in plate height was found. The exploitation of turbulence in analytical work, however, is limited by the inlet pressure requirements (probably several thousand atmospheres) for the high liquid velocities necessary to achieve turbulence in small bore columns.

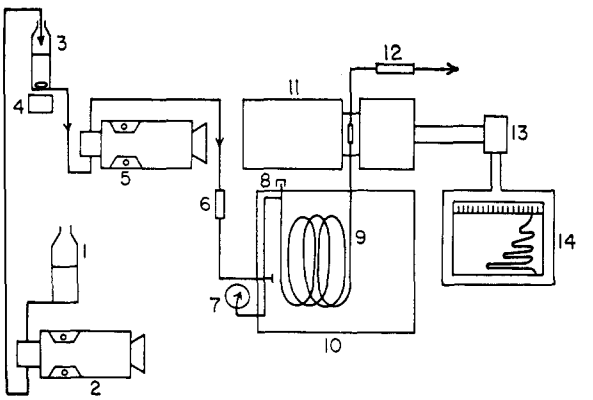

Figure 1. Liquid chromatograph with UV-detector for gradient elution

(1) Reservoir for strong eluent
(2) Pump
(3) Mixing chamber with heating
(4) Magnetic stirrer
(5) Pump
(6) Filter
(7) Pressure gauge
(8) Sample injection port
(9) Column
(10) Air thermostat
(11) Spectrophotometer with micro flow cell
(12) Flow meter
(13) Scale expander
(14) Recorder

Under laminar and turbulent flow conditions in helical open tubes radial mass transfer is enhanced by secondary flow in the cross sectional plane (*10*). Exploitation of this effect in chromatography has not been attempted as yet.

In packed columns, the fluid flows in the interstitial channels of the packing. At low flow velocities, molecular diffusion is the dominant mass transfer mechanism in the mobile phase, but with increasing flow velocity the contribution of a convective lateral mixing which depends on the packing structure becomes more significant. The result is a flattening of the H *vs.* u curve in the high velocity region. According to Giddings (*11*) the two mechanisms are coupled. He originally proposed for H_m the following expression

$$H_m = ({}^1/_2\,\lambda\, d_p + D_M/\omega\, d_p{}^2\, u)^{-1} \quad (6)$$

where λ and ω are parameters related to packing structure and d_p is the particle size. Giddings has now extended Equation 9 to a summation form which is much more general (*12*).

Knox (*13*) studied this effect in liquid chromatography using columns packed with glass beads and observed a more gradual flattening of the H *vs.* u curve in the high velocity region than predicted by the coupling theory. In liquid chromatography, owing to the low rate of molecular diffusion, coupling becomes significant at relatively low flow velocities and determines H_m over a range of several orders of magnitude in u.

In packed columns there is no sudden onset of turbulence and the decrease of h with increasing fluid velocity is not as pronounced as that in open tubes (*9*).

(4) E. W. Piel, ANAL. CHEM., **38**, 670 (1966).
(5) P. B. Hamilton, *Ibid.*, **35**, 2055 (1963).
(6) J. V. Benson and J. A. Patterson, *Ibid.*, **37**, 1108 (1965).
(7) G. Taylor, *Proc. Roy. Soc. (London)*, **219A**, 186 (1953).
(8) V. Pretorius and T. W. Smuts, ANAL. CHEM., **38**, 274 (1966).
(9) J. C. Giddings, W. A. Manwaring, and M. N. Myers, *Science*, **154**, 146 (1966).
(10) J. A. Koutsky and R. J. Adler, *Can. J. Chem. Eng.*, **43**, 239 (1964).
(11) J. C. Giddings, *Nature*, **184**, 357 (1959).
(12) J. C. Giddings, ANAL. CHEM., **35**, 2215 (1963).
(13) J. H. Knox, *Ibid.*, **38**, 253 (1966).

In the present study, the effect of various parameters on H_m in helical capillary tubes and in small bore columns packed with glass beads was investigated. The results suggested the use of glass beads coated with a thin skin (pellicle) of stationary phase in a packed column. The rapid separation of nucleoside phosphoric acids was then achieved by utilizing ion exchange resin-coated glass spheres.

EXPERIMENTAL

Figure 1 shows a block diagram of the instrument used. For unsorbed solutes, a differential refractometer (Emmett Watson Associates, Ridgefield, Conn.) having a cell volume of 7 μl was used as detector.

For nucleotides, the detector was a Perkin-Elmer–Hitachi UVIS 139 spectrophotometer modified to accommodate a specially designed micro flow cell having a volume of 8 μl and 4-mm path length. With a scale expander, a full scale signal (1 mV) could be obtained at 0.04 absorbance unit. In this very low absorbance range, at constant flow rate and buffer composition, peak areas were considered to be linearly dependent on the amount of solute present. Analyses were performed at a wavelength of 260 mμ and slit width of 2 mm.

The fluid was forced through the system by a Milton Roy Minipump which provided leak-free operation at pressures up to 4000 psi in the low capacity range (0–20 ml per hour). A 300-ml glass cylinder equipped with an immersion heater and magnetic stirrer served as a solvent reservoir. For gradient elution, a second Milton Roy Minipump was employed to add the stronger eluent to the weaker eluent placed in the reservoir. In certain experiments a Greer Accumulator (Greer Olaer Products, Los Angeles, Calif.) was used to generate nonpulsating flow.

The temperature of the system was controlled by a Statham thermostated chamber, Model SD10-1. A 0–10,000 psi Helicoid gauge with "capillary bleed" was used for monitoring column inlet pressures. In the continuous flow mode, sampling was performed with a modified Loenco sampling valve. In the intermittent flow mode, a 10-μl Hamilton syringe was used to inject the sample onto the column.

The pellicular ion exchange resin used in this study was prepared as follows. Glass beads ($d_p \lesssim 50$ microns) were coated with a solution of styrene, divinylbenzene, and benzoyl peroxide in ether and the solvent was evaporated. Polymerization and crosslinking were carried out at 90° C in an aqueous suspension of the coated beads. The product was washed with water, acetone, benzene, and methanol, then converted into the ion exchange resin. The anion-active resin was prepared by chloromethylating the polymer and reacting the product with dimethylbenzylamine. The pellicular ion exchange resin was then sieved and washed with 2M NaOH and HCl solutions and dried.

Although the use of this resin only is discussed, other resins were prepared and also found to be useful. For example, cation-active resin was prepared by sulfonating the coating of glass beads ($d_p \approx 50$ microns) with 98% sulfuric acid at 90° C for 30 minutes. For making crosslinked polyethylene-imine resin, the water from commercial polyethylene-imine (Dow Chemical Co.) was removed by distillation with benzene. Glass beads ($d_p \approx 100$ microns) were coated with a solution of the dry polyethylene-imine in methylene chloride and the solvent was evaporated. The coated beads were then exposed to methylene bromide vapor at 130° C for 10 minutes in order to crosslink the polymer. The product was washed with acetone, water, diluted hydrochloric acid, then with water, and dried.

The dry column material was packed into straight stainless-steel (No. 316) tubing (1-mm i.d., 0.0625-inch o.d.) by vibration and tapping. The outlet of the column was plugged with a thin disk of porous Teflon. To consolidate the packing, deaerated mobile phase was pumped through the column in perpendicular position for 5–6 hours, at inlet pressures of 3500–4000 psi. This treatment caused the packing to contract a few centimeters. The column was then coiled and connected to the instrument.

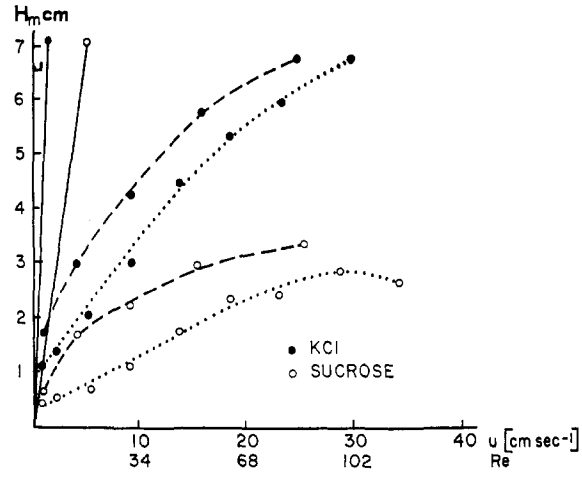

Figure 2. Plot of H_m against flow velocity in helical capillary tubes

Unbroken lines, calculated for straight tube; broken lines, Column I; dotted lines, Column II

After resieving, glass microbeads (Microbeads Inc., Jackson, Miss.) were soaked in chromic sulfuric acid overnight, washed with water and methanol, and dried. Although reagent grade chemicals were used for the preparation of buffers, some were contaminated with ultraviolet absorbing materials which gave rise to a significant base line drift during gradient elution. Reference mixtures were prepared by dissolving the purest available nucleotides in water. Perchloric acid extracts of mouse brain and liver were made in the usual manner and concentrated by lyophilization.

The linear gradient used in these analyses was produced by maintaining the flow rate through the column twice as high as the flow rate of the stronger eluent into the reservor. Samples were injected with a microsyringe at intermittent flow. After closing the sampling ports, both pumps were started at the same time; however, the sample was first eluted with the weak eluent which filled up the volume of the system between the reservoir outlet and the column inlet (approximately 1.7 ml). After each analysis the column was regenerated with approximately 15 ml of liquid. Thus each run was preceded by a 25-minute reconditioning period. The column was used over 5 months with various acidic and neutral salt solutions and occasionally with aqueous ethanol. Nevertheless, retention times remain reproducible on the properly reconditioned column.

RESULTS

Study of Peak Broadening in Columns without Stationary Phase. To assess the significance of secondary flow (*10*) in capillary columns, solute band spreading in coiled uncoated open tubes was investigated. The dimensions of the two helical columns (I and II) made of thin-walled stainless-steel tubing were as follows: Column I was 0.4-mm o.d. by 0.276-mm i.d. with a helix diameter (D_h) of 85 mm. The D_h/i.d. was 308. Column II was 0.4-mm o.d. by 0.287-mm i.d., D_h 1.28 mm, and the D_h/i.d. 4.5. The plots of plate height (H_m) *vs.* flow velocity (u) obtained by injecting KCl and sucrose as tracers in water onto these columns at 38° C are depicted in Figure 2.

Peak dispersion in Column I (broken lines) and Column II (dotted lines) is less than calculated by Equation 5 (unbroken lines) but nevertheless unacceptably large for chromatography.

Instead of the linear increase of H with laminar flow velocity, as predicted by Taylor and Golay, the H *vs.* u curve tends to flatten at high flow velocities. Contrary to the existing belief in chromatography, a decrease in the helix-diameter-to-column-inside-diameter ratio results in a decrease in plate height.

The higher plate heights obtained in both columns for the more slowly diffusing sucrose indicates that molecular diffusion plays a significant role in mass transfer under these conditions.

Although flattening of the H *vs.* u curves in coiled open tubes as shown in Figure 2 was expected as a consequence of secondary flow (*10*), the relatively small effect of coil-to-tube-diameter ratio in comparison to the strong effect of diffusivity suggests that other mechanisms cooperate in reducing H/u. It was thought that the roughness of the inner wall of the tube or the pulsating character of the flow might be responsible for the departure from the parabolic flow pattern. The wall effect is very difficult to assess but microscopic examination of the tube interior revealed no unusual roughness. On the other hand, Aris (*14*) proved theoretically that the effect of pulsation on peak dispersion in open tubes at laminar flow is insignificant unless the mean pressure gradient is smaller than the amplitude of the pressure gradient fluctuations. This is in good agreement with our findings with packed columns under the present experimental conditions because neither the pulsating nor the nonpulsating character of the flow had any demonstrable effect on plate height or retention times.

Although peak dispersion in open capillary tubes continues to be an interesting theoretical problem, the use of open tubular columns under these circumstances does not appear to be a promising approach to high speed liquid chromatography because the H_m/u values calculated from data shown in Figure 2 are much higher than those in gas chromatography.

In order to find optimal conditions for microanalysis, band spreading in two 150-cm long by 1-mm i.d. columns packed with uncoated glass beads was investigated. The small tube diameter was chosen to lessen trans column effects detrimental to column performance (*15*). The contribution of the injection and detector system to peak broadening was negligible in these experiments.

In the tracer experiments (Figure 3) with these columns, solutes of various diffusivities were used in order to observe the effect of particle size, flow velocity, and molecular diffusion on peak spreading. Thus, 10 μg of KCl (D_m = 2.43 $\times$ 10^{-5}), sucrose (D_m = 7.25 $\times$ 10^{-6}), and albumin (D_m = 7 $\times$ 10^{-7}) were injected in the carrier stream of deaerated water. The column temperature was 38° C and flow was laminar. No variation in plate height was found with decreasing the sample size down to 2 μg.

With Column B the flattening of the H_m *vs.* u curves becomes significant at flow velocities of about 0.2 cm second^{-1}. As predicted by the coupling theory, the curves converge at high flow velocities indicating an increasing contribution to lateral mass transfer from convection and decreasing contribution from molecular diffusion. With Column A the plate height is significantly greater relative to the particle diameter than expected from data from the chemical engineering literature or from that published by Knox (*13*). The discrepancy is attributed to the small particle and tube sizes used in this study compared to those described in the literature.

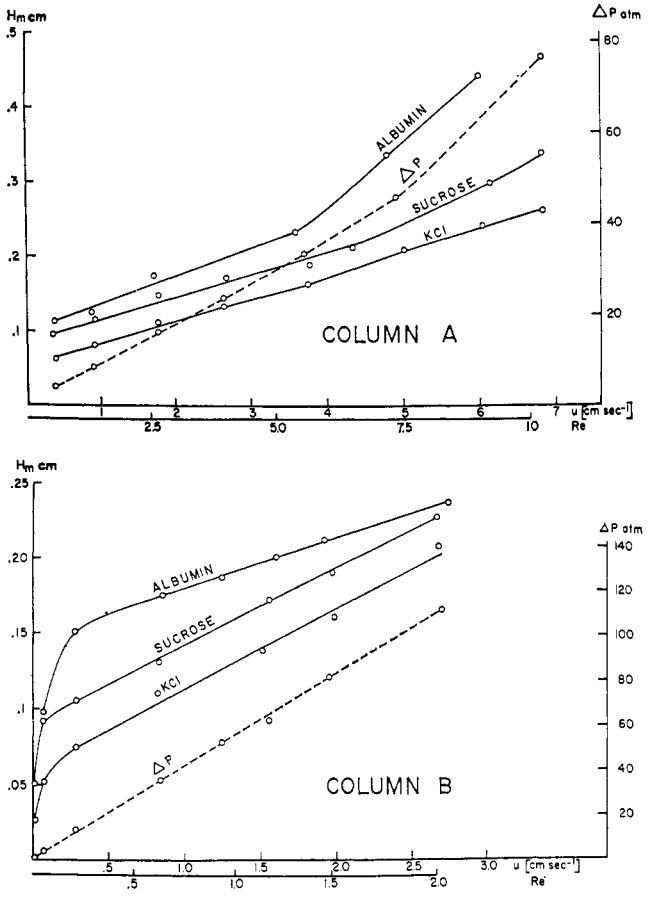

Figure 3. Plots of H_m and pressure drop against flow velocity in Column A and Column B

Column parameters were: Particle size, 88–105 microns (A), 44–53 microns (B). Δp per unit length at u = 1 cm second^{-1} (water 38° C) 0.064 atm cm^{-1} (A), 0.27 atm cm^{-1} (B). Porosity 37.9% (A), 39.8% (B). Column to particle diameter ratio 10(A), 20(B)

In chromatography, however, a relatively large surface area of the support material is needed for the fine distribution of the stationary phase. The surface area of glass spheres is inversely proportional to the diameter. Thus, the layer thickness of a given amount of stationary phase in the column decreases with the particle diameter. Because the plate height contribution of the stationary phase is proportional to the square of the layer thickness, a small particle size is attractive to reduce this term. On the other hand, the flow resistance of the column decreases with the square of the particle diameter; therefore, the available inlet pressure limits particle size reduction.

The particle size range selected for these experiments represents a practical compromise for chromatographic application. In both columns A and B, H_m/u decreased with flow velocity but appeared to approach a minimum value at the highest velocities, although significant reduction was achieved by increasing flow velocity to 1–2 cm second^{-1}. A further increase in u gave only marginal improvement in H_m/u with a large increase in the inlet pressure. Thus, $1 < u < 2$ cm second^{-1} seems a reasonable compromise velocity, comparable in its practical significance to the optimum practical flow velocity defined by Scott (*16*) in gas chromatography.

(14) R. Aris, *Proc. Roy. Soc.* (*London*) **259A**, 370 (1960).
(15) A. B. Littlewood, "Gas Chromatography 1964," A. Goldup, Ed., The Institute of Petroleum, London, 1965, pp. 77–93.
(16) R. P. W. Scott and G. S. F. Hazeldean, "Gas Chromatography 1960," R. P. W. Scott, Ed., Butterworths, Washington D. C., 1960, pp. 144–61.

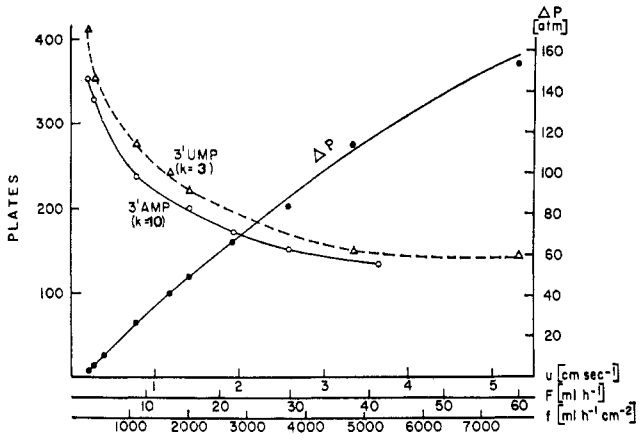

Figure 4. Plate number and pressure drop as a function of flow velocity and flow rate obtained with the pellicular anion exchanger column

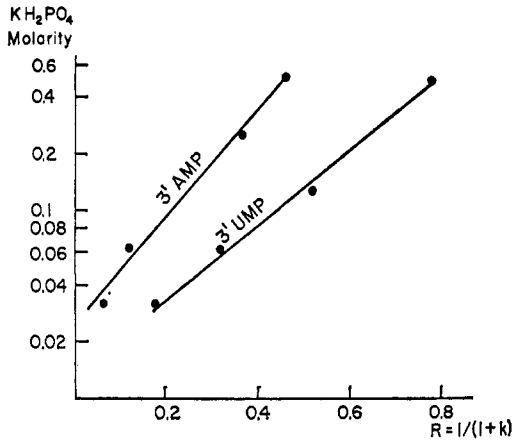

Figure 5. Effect of salt concentration on retention at 60° C

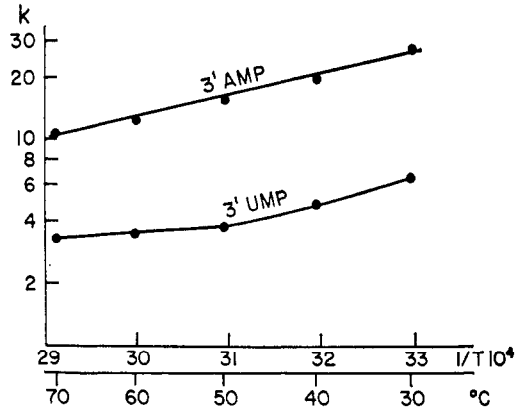

Figure 6. Effect of temperature on retention

Mobile phase: 0.045M KH_2PO_4, 0.002N H_3PO_4

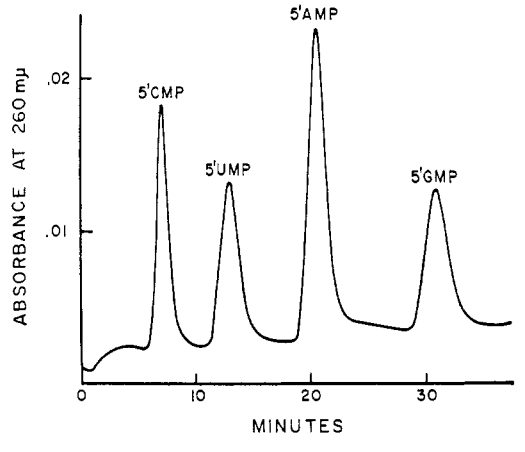

Figure 7. Separation of ribonucleoside-5'-monophosphoric acids

Column: 1-mm i.d., 193-cm length; pellicular strong anion exchange resin, sieve fraction No. 270–325, 8 microequivalent per gram. Eluent: Linear gradient of KH_2PO_4 from 0.025M to 0.325M (starting solution 2.5 × $10^{-3}N$ for H_3PO_4). Temperature: 70° C. Flow rate: 24 ml per hour. Flow velocity: 2.1 cm per minute. Pressure: 75 atm. Sample: 1 μg of 5'CMP, 5'UMP, 5'AMP, 5'GMP

The comparison of open tubular and packed columns indicates that the latter are superior for liquid chromatography. First, more favorable H_m/u values are obtained with packed columns than with open tubes. Second, because of their higher sample capacity and performance, a higher concentration of the emerging solutes is obtained with small-bore packed columns than with open tubes. This was particularly important with respect to the sensitivity of presently available detection systems.

Performance of a Pellicular Ion Exchange Column. For separation of nucleotides [ribonucleoside monophosphoric acids (2′ and 3′ or if not indicated, 5′): CMP (cytidylic acid), UMP (urydylic acid), AMP (adenylic acid), GMP (guanylic acid); ribonucleoside diphosphoric acids: CDP, UDP, ADD, GDP; ribonucleoside triphosphoric acids: CTP, UTP, ATP, GTP], a 193-cm long, 1-mm i.d. column was packed with No. 275-325 sieve fraction of glass beads coated with an anion exchanger containing benzyl-dimethylammonium groups. The capacity of the resin was 8 microequivalents per gram as determined by the method of Helfferich (*17*). Figure 4 shows plots of plate number and pressure drop against the linear flow velocity for 3′UMP and 3′AMP. The column capacity ratios (k) for these solutes were 3 and 10, respectively, large enough for the plate numbers to reflect the resolving power of the column. The flow rate, F, and the flow rate per square centimeter, f, are indicated in Figure 4 to allow comparison of our flow conditions with those in other liquid chromatographic systems. The retention time of an unsorbed solute was measured with cytosine, which was not significantly retarded under the conditions of the experiment. The plate numbers are 50–100 times smaller than those measured with unsorbed solutes because of the stationary phase in the column, and consequently with 3′AMP H/u values were 1–2 orders of magnitude greater. Again, it was not economical to increase the flow velocity above the practical optimum range, as a small additional increase in speed of analysis requires a large increase of inlet pressure. Therefore, for analytical work with this column, the flow velocity was fixed at u = 1.2 cm $second^{-1}$.

Factors Affecting Retention. Because the composition of the mobile phase greatly influences the behavior of solutes in the stationary phase, buffer solutions other than those utilized in this study might provide better column performance

(17) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, 1962, pp. 91–2.

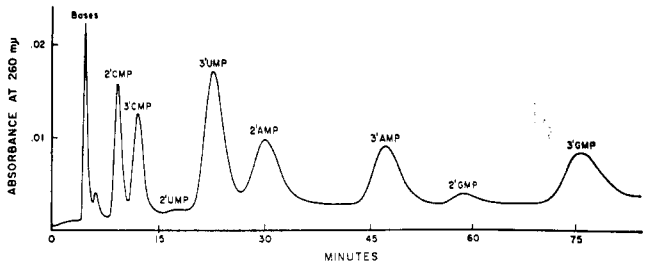

Figure 8. Separation of ribonucleoside-2′ and 3′-monophosphoric acids

Column: 1-mm i.d., 193-cm length; strong basic pellicular anion exchanger, sieve fraction No. 270–325, 8 microequivalent per gram. Eluent: linear gradient of KH_2PO_4 from 0.05M to 0.35M (starting solution 0.005N for H_3PO_4). Temperature: 60° C. Flow rate: 12 ml per hour. Flow velocity: 1 cm second^{-1}. Pressure: 51 atm. Sample: 1 μg of each isomer mixture

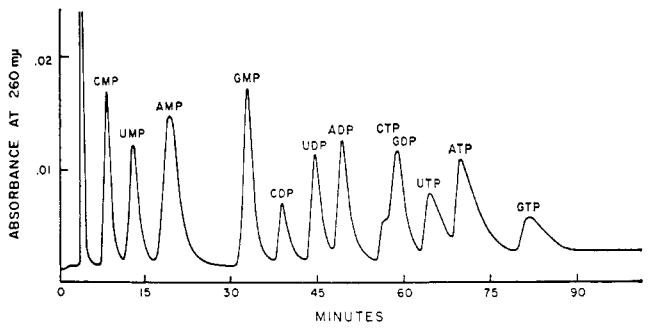

Figure 9. Separation of ribonucleosides mono-, di-, and triphosphoric acids

Column: 1.0-mm i.d., 193-cm length; packed with pellicular anion exchanger (strong basic), sieve fraction No. 270–325; 8 microequivalent per gram. Eluent: linear gradient of ammonium formate buffer, pH 4.35 at 25° C, from 0.04M to 1.5M. Temperature: 71° C. Flow rate: 12 ml per hour. Flow velocity: 1 cm second^{-1}. Pressure: 51 atm. Sample: 1.5–3.5 nanomoles (0.6–1.5 μg) per component

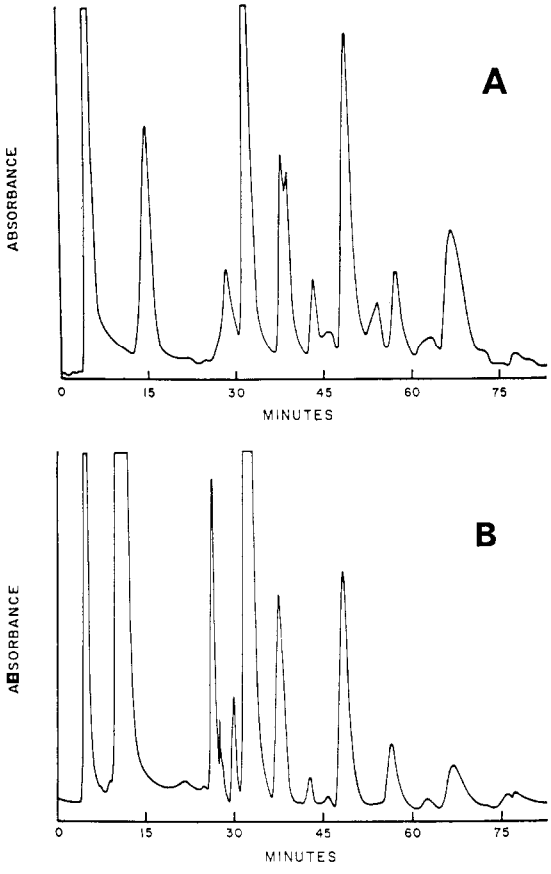

Figure 10. Separation of cellular extracts from mouse liver (*A*) and mouse brain (*B*) on pellicular ion exchanger using linear gradient elution with ammonium formate buffer, pH 4.35

and peak symmetry, but, at present, no good guide exists for the optimization of the composition of the mobile phase.

The effect of salt concentration C on the retardation factor, R, is shown in Figure 5 for the elution of 3′AMP and 3′UMP with KH_2PO_4 solutions of different concentrations. The curves correspond to the equation

$$R = a \log C + b \qquad (7)$$

where a and b are constants.

The column performance improved with temperature due to increased solute diffusivity in both phases. At high temperatures, a significant reduction in tailing was also observed. Thus, rapid analysis can be achieved at the highest temperature tolerable for the solutes, the stationary phase, and the mobile phase.

Because increase of temperature reduces k, (see Figure 6), this combined with improvements in speed may enable many compounds conventionally analyzed at relatively low temperatures and residing in the column for many hours to be separated at higher temperatures without decomposition. Thus, although nucleotides (particularly nucleoside di- and triphosphates) are relatively labile substances, no decomposition was observed in our work during the chromatographic runs at 70° C.

Columns packed with pellicular ion exchange resins are characterized by a large ratio of mobile phase to stationary phase compared to columns packed with the conventional ion exchange resin. Thus, the eluent strength of the mobile phase can be much lower in the pellicular ion exchange column than in the conventional column in order to obtain the same mass distribution ratio.

Nucleotide Separation. Nucleotides were among the first biochemical substances to be separated by ion exchange chromatography (*18*). A major advance was made by Anderson and his colleagues (*19*) with the development of automated ion exchange chromatography similar to that used for amino acid analysis (*20*). Although rapid separation of relatively simple nucleotides can be achieved by thin layer chromatography (*21*), the analysis of nucleic acid hydrolyzates is most commonly carried out by means of conventional column chromatography on a strong anion exchanger polystyrene resin. This method usually requires at least 20 hours to separate the nucleoside mono-, di-, and triphosphates. Moreover, the column system is not reusable and relatively large samples are necessary.

Recently, automated ion exchange chromatography of amino acid and peptide mixtures has been greatly accelerated by introducing uniform spherical ion exchange resins having small particle size (*6*) and extended for the analysis of nano-

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(19) N. G. Anderson, J. G. Green, M. L. Barber, and SR. F. C. Ladd, *Anal. Biochem.*, **6**, 153 (1963).
(20) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).
(21) K. Randerath, "Thin Layer Chromatography," Academic Press, New York, 1963, pp. 188–99.

mole quantities of amino acids by using small-bore columns. Recent work of Cohn and Uriel (*22*) shows that the analysis of nucleic acid bases and nucleosides at the nanomole level can be achieved on columns packed with a fine resin at increased detector sensitivity.

Similar modifications must improve the speed and sensitivity of analysis of nucleotides. However, the use of conventional ion exchange resins is beset by some inherent difficulties. Large differences in the equilibrium constants for the mono-, di-, and triphosphate nucleotides require a strong gradient of the eluent: the accompanying shrinking and swelling causes undesirable changes in column permeability and column performance. Ion exchange resins contain low molecular weight contaminants which give rise to column bleeding, which is undesirable when one wishes to collect eluted compounds and which causes base line drift with ultraviolet detectors.

These difficulties are overcome by the use of pellicular ion exchange resins which also permit the fast analysis of minute quantities of nucleoside phosphoric acids. Figures 7–9 show chromatograms of nucleotide mixtures analyzed by this liquid chromatography system using gradient elution with different buffer solutions. The analysis time compares well with that for gas chromatography. Because the order of elution is the same as that with Dowex-1 columns, the retention mechanism for the classical and pellicular ion exchange resin is probably the same.

(22) W. E. Cohn and M. Uriel, Oak Ridge National Laboratory, Oak Ridge, Tenn., personal communication, July 14, 1966.

The efficiency of this complete system is also demonstrated by the chromatograms of cellular extracts of mouse brain and liver in Figure 10. Although the identification of the individual peaks has yet to be completed, the chromatograms indicate more components to be present than are revealed by conventional separation methods.

Of the other pellicular resins mentioned above, pellicular polyethyleneimine resin was useful for the fast separation of nucleic acid bases and nucleosides. Pellicular alumina and carbon black were also effective in the separation of lipids. Column efficiency and optimum operating conditions were similar to those described in this study.

In conclusion, the use of pellicular column materials in small bore columns are widely applicable for liquid chromatography. The stability and efficiency of such materials make it possible to achieve fast separation of complex mixtures by a liquid chromatographic technique similar in speed, resolution, and quantitative range to gas chromatography.

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Design of a Nickel-63 Electron Absorption Detector and Analytical Significance of High Temperature Operation

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An electron absorption detector which incorporates a ^{63}Ni source has been designed for safe operation to 400° C. The analytical advantages of high temperature operation have been studied so as to extend the general utility of this class of detector in gas chromatography. Expansion of the practical temperature range reduces detector contamination and provides a broader insight into the temperature dependency phenomena observed for certain compounds. Moreover, the new design retains all of the desirable performance characteristics of the established Lovelock parallel plate electron capture detector when operated in the pulsed sampling mode.

SINCE ITS INCEPTION in 1960 (*1*), the electron absorption detector has enjoyed a unique position as a remarkably sensitive and highly selective detector for gas chromatography. The precipitous acceptance of this detector as a powerful adjunct to gas chromatography tended to obscure some of the errors inherent in its earlier designs and modes of operation. In 1963 Lovelock described the plane parallel design which, when operated with pulse collection potential, substantially freed the device from anomolous responses and errors induced by a continuous electrical field (*2*).

Wentworth *et al.* (*3*) applied a kinetic model to the study of electron attachment phenomena occurring in the pulse-sampled parallel plate detector, which has considerably expanded our knowledge of the fundamental physical processes involved. The merit of this approach may be judged from the successful calculation of electron affinities and bond dissociation energies for a number of compounds based on information obtained through the use of the detector at various temperatures (*4–7*). These studies had significant analytical importance, in that a linear relationship between detector response and sample concentration was derived which was

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