Journal of Chromatography, 124 (1976) 187–196

CHROM. 9264

SIMPLIFIED LOW-PRESSURE HIGH-RESOLUTION NUCLEOTIDE ANALYSER*

D. PERRETT

Medical Unit, St. Bartholomew's Hospital, London EC1A 7BE (Great Britain)

(Received February 12th, 1976)

SUMMARY

A simple nucleotide analyser was constructed from commercially available components. A 20 cm × 0.4 cm I.D. column of pellicular anion exchanger operating at approximately 20 p.s.i. was found to perform identically with published methods using high-pressure equipment. The eluant was monitored using a variable-wavelength UV monitor. A very simple device for producing variable gradients was employed. The nucleotide analyser was trouble free to operate and the results were both linear and reproducible. Applications to the rapid analysis of nucleotides and the high-sensitivity analysis (approx. 10 pmoles) of cyclic 3′,5′-adenosine monophosphate and cyclic 2′,3′-guanosine monophosphate and tissue nucleotides are described.

INTRODUCTION

Modern high-performance/high-resolution liquid chromatography (HPLC) has developed rapidly over the last few years with the introduction of high-efficiency packings, improved detectors, and improved understanding of the processes involved. Commencing some few years earlier and generally following an independent course, the automated amino acid analyser has been developed into the specialised liquid chromatographic (LC) system "par excellence". Much of the work on amino acid chromatography, although applicable to LC in general, has been overlooked in the recent growth of HPLC. In order to study nucleotide metabolism in cells, accurate quantitation of nucleotide concentrations was required. Therefore, a nucleotide analyser simpler than the presently available commercial models has been developed by employing principles originally outlined for amino acid analysis.

Present nucleotide analysers (for review, see ref. 1), developed from the work of Horvath *et al.*[2] on pellicular resins, typically employ stainless-steel columns of up

* Abbreviations: AMP, ADP, ATP, GMP, GDP, GTP = adenosine and guanosine 5′-mono-, 5′-di- and 5′-triphosphates, respectively; cAMP = cyclic 3′,5′-adenosine monophosphate; cGMP = cyclic 2′,3′-guanosine monophosphate; CMP, CDP = cytidine 5′-mono- and 5′-diphosphate; TMP, TDP = deoxythymidine 5′-mono- and 5′-diphosphate; NAD, NADP = nicotinamide-adenine dinucleotide and phosphate; UMP, UDP = uridine 5′-mono- and 5′-diphosphate; IMP = inosine 5′-monophosphate; TCA = trichloroacetic acid.

to 3 m in length and 1 or 2 mm I.D. and develop pressures up to 3000 p.s.i. at flow-rates of 1 ml/min. In order to utilize previously existing low-pressure equipment from an amino acid analyser as a nucleotide analyser, a system which operates at substantially lower pressures was desirable. Mondino[3], following experimental studies on the role of column dimensions in amino acid analysis, stated that provided the correct volume of resin for the separation under investigation was employed, the resolution and the sensitivity of the system were apparently independent of the column dimensions. It was therefore decided to investigate if this principle could also be applied to nucleotide analysis.

Using the dimensions of the nucleotide analyser of Brown[4], *i.e.*, a 3 m × 1 mm column of pellicular anion exchanger as typical, a nucleotide analyser with a column of the same volume (*e.g.*, 2.35 ml) but only 20 cm × 4 mm I.D. has been constructed and evaluated.

METHODS

Apparatus

A 20 cm × 4 mm I.D. glass column (Jobling, Stone, Great Britain) was dry packed with AS-Pellionex-SAX (Reeve Angel Scientific, Maidstone, Great Britain) to a height of 19 cm. No top column sinter was employed but the resin bed was protected by two layers of glass fibre filter paper. The column was fitted with a septum injector and was maintained at constant temperature by a circulating water jacket. All column fittings were obtained from Jobling.

A Milton Roy minipump was initially employed but for high-sensitivity work (below 0.05 a.u.f.s.) the pulse-damped version was used.

The column eluant was monitored by a CE212 variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) equipped with a 10-μl flow cell and a CE213 auto-range unit, which reduces off-scale peaks by set factors back onto the recorder scale. The output from the monitor was recorded on a Servoscribe potentiometric recorder which possessed facilities for expansion up to 0.05 mV f.s.d. For high-sensitivity work the output of the CE212 monitor was filtered using a simple RC filter as described by Brooker[5]. The column base was connected to the flow cell by the shortest possible length (25 cm) of 0.3 mm I.D. PTFE tubing. The conductivity of the column eluant was monitored by a simple flow cell consisting of two 1-cm pieces of 21G stainless-steel needle separated by a sleeve of rigid polythene tubing so that there was a gap of approximately 5 mm between the two pieces. Leads from the two pieces of stainless steel were fed to a simple battery-operated conductivity meter (Model CM25 WPA; Saffron, Walden, Great Britain) and when necessary the output was recorded on a 0–10 mV recorder. When required, the column eluant was collected using a time-based fraction collector (Chemlab, Ilford, Essex, Great Britain), the sample tray of which was modified to accept 6-ml plastic scintillation tubes (Sterilin, Teddington, Great Britain).

The construction of the gradient former used in this method is detailed in Fig. 1. It consists of a flexible length of solid polythene tubing sandwiched between two 10-cm squares of 1/8-in. perspex sheet separated on three sides by 0.7 × 10 cm lengths of 1/8-in. perspex. The whole, including the lower end of the tubing, was firmly sealed with plastic cement. Two 2-mm holes were drilled to take small lengths of PTFE

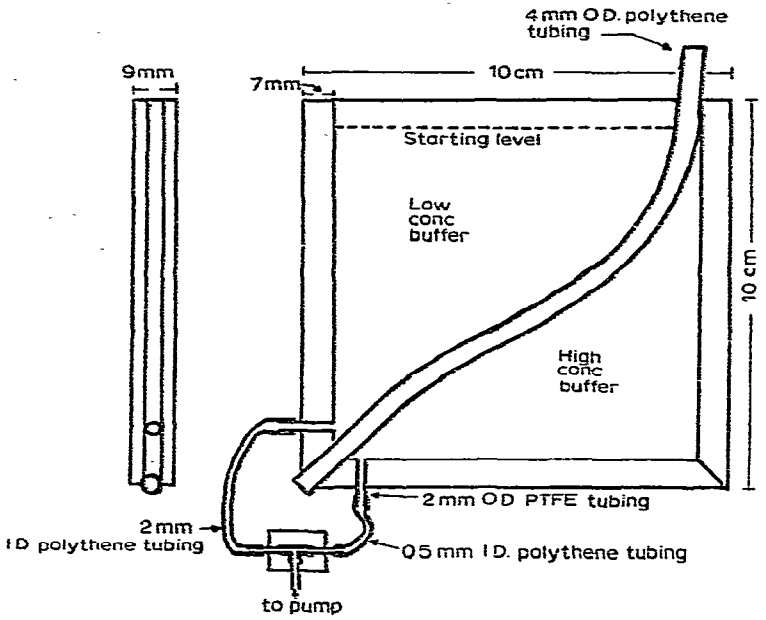


Fig. 1. Construction of the gradient former. The body is composed of $^1/_8$-in. perspex sheet.

tubing, as shown. A Technicon micro-T piece was connected to the two outlets by lengths of autoanalyser tubing. In order to prevent an initial unbalanced loss from the high-concentration chamber, it was found necessary to restrict the flow from that chamber by the use of narrower-bore tubing, as shown. The gradient produced was approximately identical with the shape of the plastic curve. This could be straightened by a gentle pull at the free end and then moved to any required shape by application of gentle pressure on the top surface of the tubing with a stiff piece of suitable metal rod. The tubing formed a water-tight seal between the two chambers. For future reference the shape of any particular gradient could be marked on the outer surface of the gradient former. In order to avoid too much disturbance to the gradient is was convenient to fill the high-concentration chamber using a syringe, the needle being inserted into the chamber. The working capacity of the gradient former described was approximately 22 ml. The gradient former was mounted in a vertical position with the liquid level horizontal.

In operation the connection between the T piece and the high-concentration chamber was sealed by a clip on the polythene tubing and low-concentration chamber buffer alone was pumped until the column was equilibrated. Both chambers were then filled to the starting level and the gradient started by removing the clip. No mixing chamber was required as sufficient mixing was provided by the action of the pump. The gradient so formed was found to be very reproducible.

In order to minimize the dead time between the opening of the gradient former and the arrival of the gradient at the top of the resin bed, the shortest necessary lengths of 0.58 mm I.D. PTFE connecting tubing were employed. The dead volume of the system was determined by injecting a sample of concentrated sodium chloride solution onto the column, immediately starting the gradient and measuring the time between

the injected peak and the appearance of the gradient on the conductivity meter. The dead volume was calculated to be 1.1 ml.

Chemicals

All commercial samples of potassium dihydrogen phosphate or chloride tested were found to possess high UV absorption below 270 nm and this prevented satisfactory operations of a gradient elution system at high sensitivity. In order to utilize the high sensitivity of the detector, it was necessary to purify both salts by recrystallisation of laboratory grade reagents. The potassium chloride was recrystallised twice in the normal manner. As the UV absorbing impurities occurring in the phosphate are concentrated in the crystals, the supernatant obtained after crystallisation was further concentrated and recrystallised. This was repeated until a satisfactory material had been obtained. Another source of serious baseline rise was traced to UV absorbing material in the de-ionised water of the laboratory and therefore only freshly de-ionised water was employed.

Standard solutions (3 m*M*) of nucleotides (Sigma, St. Louis, Mo., U.S.A.) were prepared in de-ionised water and stored at −20°. Suitable composite solutions (approx. 0.5 m*M*) were prepared for use when required.

Because of the increasing salt concentrations with gradient elution in order to prevent unstable mixtures, it was found necessary to use an emulsifying scintillant for radioactivity counting. 2.5 ml of Unisolve I (Koch-Light, Colnbrook, Great Britain) was added directly to the column eluant fractions (approx. 1 ml) collected in the 6-ml scintillation tubes. The counting efficiency of this mixture did not vary with the salt concentration and was found to be 60%.

Operation

Samples (up to 30 μl) could be satisfactorily injected directly onto the resin bed without stopping the flow using standard microlitre syringes (SGE, London, Great Britain) equipped with 7-cm needles sleeved so that the tip just reached the top of the resin bed. For the gradient elution of nucleotides the flow-rate was usually 22 ml h^{-1} and the pressure generated was then below the 20 p.s.i. mark on the pressure gauge of the pump. At these very low pressures difficulty with air bubbles in the cell was encountered and to overcome this the whole system was back pressured (to 40 p.s.i.) using a screw clip to compress the polythene tubing between the monitor and the conductivity cell. Regeneration of the column could be accomplished in a few minutes by simply increasing the pump rate to about 70 ml/h without creating excessive pressures. The column could also be cleaned by direct injection of 500 μl of 1 *M* hydrochloric acid into the system. The pump was used in a partially damped mode only. Peak areas were measured by the height multiplied by half-width method.

After about forty chromatograms the septum and the glass fibre filters were changed. Resolution was found to slowly deteriorate, particularly when biological samples were being analysed and after two months (*i.e.*, 200 runs) the column was emptied and the packing cleaned. The top third of the packing, which was usually stained noticeably brown, was rejected and the remainder was cleaned with 1 *M* potassium hydroxide, followed by water, 1 *M* hydrochloric acid, water, and finally acetone before allowing it to dry. The column was then repacked using new resin to replace the losses. This procedure was found to restore the resolving power of the column completely.

EXPERIMENTAL AND RESULTS

Because of the difficulties associated with the reproduction of gradients, the present system was initially evaluated by comparison of isocratic elution chromatograms with those of other authors using long-column high-pressure techniques.

Fig. 2 shows the separation of the adenosine nucleotides using a single buffer of 0.38 mole/l KH_2PO_4 pH 4.2 on the 20-cm column maintained at 80°. At the flow-rate of 55 ml/h the pressure developed was 50 p.s.i. The separation achieved was identical with that published by Burtis *et al.* (Fig. 7 of ref. 6) using a 3 m × 1 mm I.D. column of pellicular anion exchanger at a pressure of 2600–3000 p.s.i. Although the pump was not working in its fully damped mode, the peak-to-peak baseline noise at 254 nm did not exceed 3×10^{-4} a.u.

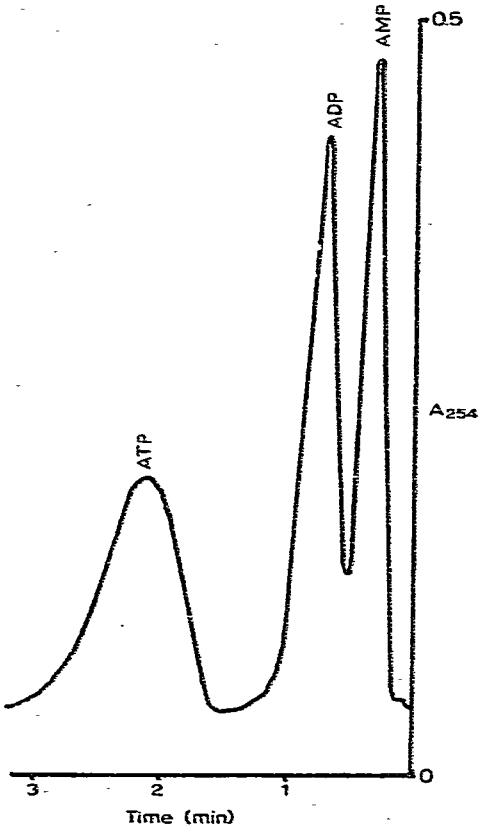
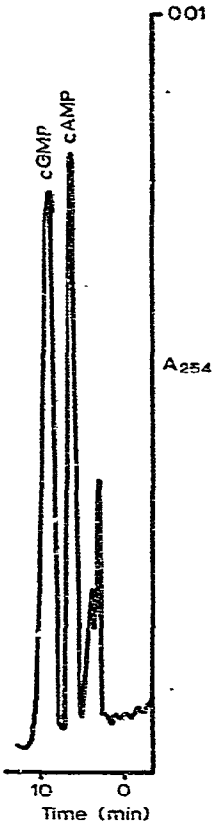


Fig. 2. Separation of adenosine nucleotides. Column, 20 cm × 4 mm I.D.; resin, AS-Pellionex-SAX; eluent, 0.38 mole/l KH_2PO_4, pH 4.2; flow-rate, 55 ml/h; pressure, 50 p.s.i.; temperature, 80°; sample volume, 10 µl; detection, 254 nm.

An initial attempt to emulate the work of Brooker[7] on cyclic nucleotide analysis was unsuccessful due to corrosion of the polypropylene column components by dilute acid. However, a 0.2 *M* KH_2PO_4 buffer was found to be capable of separating cAMP and cGMP in approximately 10 min (Fig. 3). Brooker[5] also demonstrated the applica-



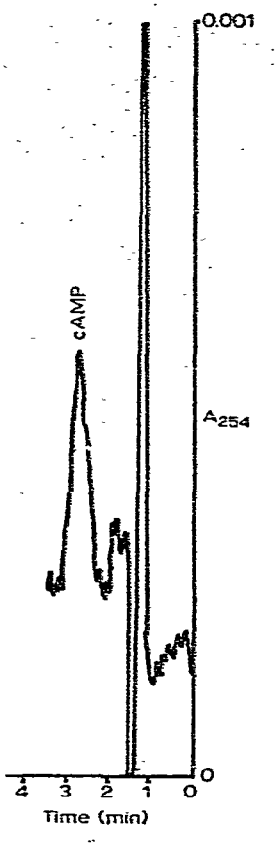


Fig. 3. Separation of cyclic nucleotides. Equipment, as for Fig. 2. Eluent, 0.2 *M* KH_2PO_4 pH 4.2; flow-rate, 20 ml/h; pressure, approx. 20 p.s.i.; temperature, ambient; sample, 200 pmoles of both cAMP and cGMP; detection, 254 nm 0.01 a.u.f.s.

Fig. 4. Separation of 10 pmoles of cyclic AMP. Conditions, as for Fig. 2, except 0.001 a.u.f.s. (recorder 0–1 mV f.s.d.).

tion of high-pressure nucleotide analysis to the determination of cAMP in the range of 10–100 pmoles by thermostating the flow cell and filtering the monitor output to remove baseline noise. Fig. 4 illustrates the operation of the present system at 0.001 a.u.f.s. The column was operated at room temperature, a simpler procedure than thermostating the flow cell, and the monitor output was fed through a 300-Kohm resistance to the recorder (range 0–1 mV f.s.d.). The peak-to-peak noise was reduced to 3×10^{-5} a.u. and 10 pmoles of cAMP could be quantitated as shown.

No single buffer system is able to separate the complete nucleotide content of cell extracts satisfactorily and for this purpose various gradients have been employed. Fig. 5 illustrates the degree of separation obtainable by the present system using the simple gradient device adjusted to give an approximately linear gradient from 0.004 *M* KH_2PO_4 pH 6.5 to 0.18 *M* KH_2PO_4 + 0.13 *M* KCl pH 4.5. As shown, the simultaneous separation of at least nine nucleotides could be easily achieved. A mixed phosphate–chloride buffer was found necessary to reduce the baseline rise due to UV

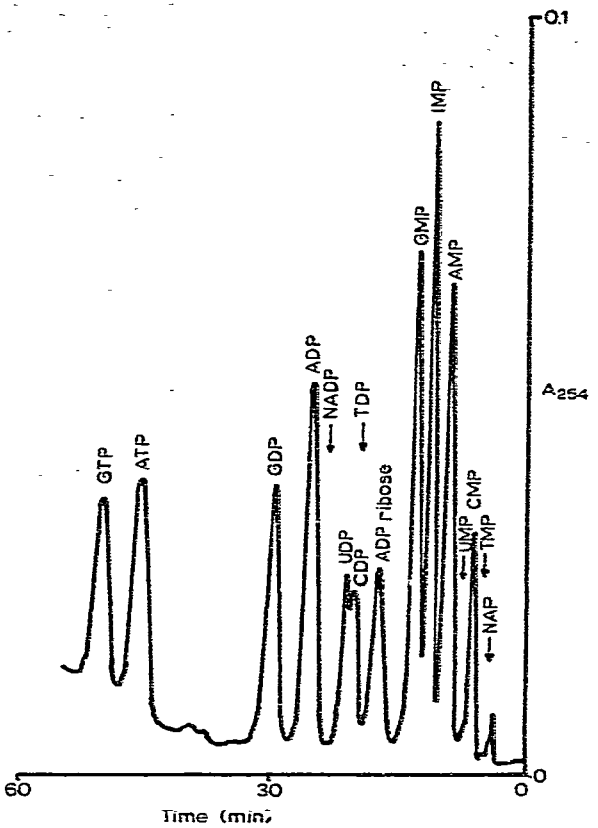


Fig. 5. Separation of nucleotide standards. Equipment, as for Fig. 2. Gradient, 0.004 *M* KH_2PO_4 pH 6.5 to 0.18 *M* KH_2PO_4 + 0.13 *M* KCl pH 4.2; flow-rate, 22 ml/h; temperature, 75°; sample, 3 nmoles of each nucleotide; pressure, approx. 20 p.s.i.; detection, 254 nm.

absorbing impurities to levels consistent with reasonable sensitivity, even so a rise of 0.01–0.02 a.u. was unavoidable.

For good separation of the mononucleotides in the early part of the chromatogram it was necessary to keep the dead volume of the system to a minimum and to raise the initial pH to 6.5 in order to position IMP between AMP and GMP. The form of the gradient could be easily modified to affect most separations, *e.g.*, careful adjustment in the middle portion was required to resolve NADP from ADP in erythrocyte analyses. The flow-rate was adjusted to give good resolution while allowing analysis and regeneration to be completed in approximately 1 h and so enabling up to eight analyses to be performed per day. The flow-rate for this system was 22 ml/h.

The effect of a number of operating parameters, *e.g.*, flow-rate, temperature, eluant composition, and pH on the gradient elution of nucleotides was investigated. Similar results to those previously reported for long-column studies[2,4] were obtained. The optimum temperature for the AS-Pellionex-SAX resin was found to be 75°; sample size, although slightly affecting resolution of complex standard mixtures, did not significantly affect analyses of biological samples; injection volumes of up to 30 μl were routinely employed.

Although most work was performed using the "standard" wavelength of 254 nm, the ability of this system to use other wavelengths can be useful. Fig. 6 illustrates the potential of employing other wavelengths for column monitoring. At 215 nm, the lowest wavelength at which the instrument could be zeroed due to the high absorption of the buffers, a different pattern of peaks relative to 254 nm was found in this TCA extract of mouse muscle. Noticeably a prominent new peak appears at 21 min, which is as yet unidentified.

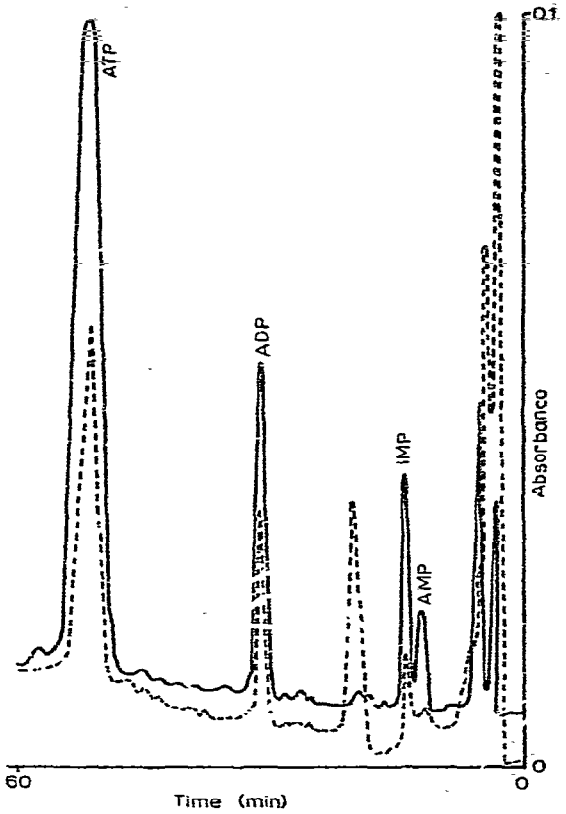


Fig. 6. Use of different wavelengths in monitoring the column eluant. Conditions, as for Fig. 5, except monitored at 254 and 215 nm. Sample, TCA extract equivalent to approximately 1 mg of mouse muscle. ———, Absorbance at 254 nm; — — —, at 215 nm.

DISCUSSION

The simple nucleotide analyser described in this paper has been in daily use for two years analysing extracts from a variety of sources, *e.g.*, erythrocytes, muscle, kidney. In that time few if any serious faults have occurred. The components of the system are readily and relatively cheaply available and spare parts can also be readily obtained. The total cost of the nucleotide analyser was less than £2,000. The fact that the operations are performed at low pressure besides simplifying the system removes the many problems associated with high-pressure pumps used on other systems.

The use of these low pressures is not entirely without problems. In particular, whereas high pressures tend to keep dissolved air in solution, in the present system air bubbles can develop in the cell, but this is easily cured as mentioned. Similar bubbles could occasionally form in the pumping chamber of the pump and thereby slightly reduce the flow-rate. It was important to check the flow-rate and when necessary adjust it before each run. As the peak width when measured in millimetres is dependent on the flow-rate for accurate peak areas the flow must be constant.

The usual methods of generating gradients in LC when only relatively small volumes are involved are either two pumps and a mixing chamber system[2] or electronic gradient makers. The present gradient device is probably more flexible than the two-pump system and certainly cheaper than either system. With care the gradient could be adjusted to achieve most separations in a reproducible manner. The capacity of the gradient former can of course be varied by changes in the dimensions of the components. Although due to surface tension effects and differences in flow characteristics from the two chambers the present small device does not entirely obey simple theory when using low volumes, it does enable reproducible gradients to be generated both simply and cheaply.

A major inconvenience in the present system is the need to purify the buffer chemicals before use. Although Shmukler[8] reported that the UV absorbing impurities could be removed by passage down an anion-exchange column, repeated attempts to duplicate this method for the present study were unsuccessful and tedious recrystallisation procedures had to be used. Even when an acceptable baseline was achieved baseline rises and artifacts could still result from a number of sources. The quality of the deionised water has already been mentioned; equally important is the condition of the resin, the top filter paper, and the septum. Due attention to these factors was found necessary to be able to run routinely at 0.1 a.u.f.s. or less. Most commercial samples of nucleotides, in particular the less common ones, were found to be relatively impure (some as low as 90%) when subjected to high-sensitivity analysis. Nucleotide analysis and probably HPLC in general often requires both standards and reagents which are purer than those presently commercially available.

The employment of a variable-wavelength monitor offers particular advantages over the fixed-wavelength units currently used. The ability to monitor at wavelengths other than 254 nm has been employed in this laboratory to study the metabolism of thiol-substituted purines, *e.g.*, 6-mercaptopurine, which absorb strongly in the 300–340 nm region. Similarly, the use of wavelength shifts can be of use as an aid to identification of peaks, *e.g.*, the AMP/IMP pair can be resolved by measuring the 250/260 ratio of the peaks. The chromatogram can either be run twice, one at each of the two wavelengths, or the chromatogram can be stopped when the centre of the peak fills the cell and measurements can be made at the two wavelengths. Similar baseline measurements before or after the peak are also required.

Mondini[5] offered no theoretical interpretation for his observations using a constant volume of crushed Amberlite IR-120 over a column length change from 21.6–11 cm. Recently Rao *et al.*[9] have employed the same pellicular resin (AS-SAX) as used in the present study in a column 1 m × 1.7 mm (volume = 2.27 ml) with results very similar to those obtained in this study. Very similar results have therefore been obtained using columns of 3 m, 1 m, and 19 cm, but of the same volume. The present study indicates that at least with pellicular anion exchangers at constant column

volume a length change by a factor of 15 from 3 m to 20 cm does not appear to effect resolution. Many studies have related such parameters as column length and column diameter to the efficiency of the chromatographic separation but as yet no full study of length and diameter at constant column volume appears to have been performed. Because this appears to offer a possible solution to the problems of high pressure in chromatographic systems, a full study using a variety of packing materials appears warranted. The only limitation on such a study at present would be the possibility that the "infinite diameter effect" of Knox and Parcher[10] would apply to some but not all the column lengths. It would also appear that the use of the term "plate height" would be misleading in this situation. For although the columns appear to possess the same resolution, *i.e.*, have an equal number of plates, the column length and therefore the plate height differs by a factor of 15.

The present system has been shown to be as reproducible as the previous high-pressure systems, having a coefficient of variation of less than 4% on standard solution of AMP, ADP and ATP compared with 3% reported by Brown[4] for AMP and 2.8–5.9%, depending on the nucleotide reported by Rao *et al.*[9]. The reproducibility of physiological extracts was found to be somewhat lower.

Recently a new class of anion-exchange packings, the microparticular chemically bonded resins, which offer improved performance over the pellicular resins, have been applied to nucleotide analysis by Hartwick and Brown[11]. Operating at room temperature in a 25 × 0.46 cm column these newer packings require relatively high pressures (*e.g.*, 1000 p.s.i.) tc produce shorter analysis times. The optimum column dimensions with these packings for nucleotide analysis remain to be investigated.

ACKNOWLEDGEMENTS

I am grateful to Dr. B. M. Dean for her encouragement and to Dr. J. R. Griffiths for preparing the muscle extract.

REFERENCES

1 P. R. Brown, *High-Pressure Liquid Chromatography. Biochemical and Biomedical Applications*, Academic Press, New York, 1973.
2 C. G. Horvath, B. A. Preiss and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1422.
3 A. Mondino, *J. Chromatogr.*, 50 (1970) 260.
4 P. R. Brown, *J. Chromatogr.*, 52 (1970) 257.
5 G. Brooker, *Anal. Chem.*, 43 (1971) 1095.
6 A. C. Burtis, M. N. Munk and F. R. MacDonald, *Clin. Chem.*, 16 (1970) 667.
7 G. Brooker, *Anal. Chem.*, 42 (1970) 1108.
8 H. Shmukler, *J. Chromatogr. Sci.*, 8 (1970) 581.
9 G. H. R. Rao, J. G. White, A. A. Jachimowicz and C. J. Witkop, *J. Lab. Clin. Med.*, 84 (1974) 839.
10 J. H. Knox and J. F. Parcher, *Anal. Chem.*, 41 (1969) 1599.
11 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.