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MEASUREMENT OF PLANT NUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Procedures are described for the chromatographic isolation of nucleotides from plant extracts and for their separation and measurement by high-performance liquid chromatography (HPLC). A plant extract containing nucleotides plus an array of other plant products and ions was adjusted to pH 2 with hydrochloric acid and passed sequentially through three small low-pressure columns containing: (1) porous polystyrene (Amberlite XAD-2), (2) water-insoluble polyvinylpolypyrrolidone, (3) charcoal–cellulose. At pH 2, the first two columns retained most aromatic compounds but not nucleotides. The latter were retained by the charcoal while inorganic ions and aliphatic compounds passed through all three columns. Nucleotides were eluted from the charcoal with ammoniacal alcohol. They were separated on an HPLC anion-exchange column with gradient elution. During the passage of a dilute phosphate mobile phase through the column, large changes in pH occurred because of Donnan equilibrium effects. These effects prevented pH equilibration of the column in a reasonable time, caused irregularities in nucleotide retention time, and even changed elution order. The addition of 0.01 *M* acetate to a dilute phosphate mobile phase improved pH control so that column pH equilibration was achieved within 50 min. Nucleotide retention times were stabilized and separation improved. At 0.01 *M*, the acetate showed relatively little tendency to compete with phosphate for exchange sites.

INTRODUCTION

Nucleotide concentrations and ratios in cells and tissues are useful indicators of energy status and metabolism[1–3]. At present, one of the most efficient procedures for the separation and measurement of nucleotides is by anion-exchange high-performance liquid chromatography (HPLC) with ortho phosphate (Pi) gradient elution[4–9].

In the past, two related problems have limited the application of this procedure to plant nucleotides: (1) Plant tissues contain low concentrations of nucleotides and relatively high concentrations of ions, phenols, and other secondary plant products[10,11] that interfere with the separation and measurement of nucleotides. Some of the interfering compounds rapidly poison an analytical chromatographic column. (2)

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Many of the compounds present in the nucleotide fraction of plant extracts are only weakly bound on an anion-exchange columnn. To separate these compounds it is necesssary to begin the elution with a low concentration of Pi, which does not control the pH of the mobile phase.

Frequently the pH of the mobile phase is tacitly assumed to be the same on the column as in the reservoir. However, as Singhal[12] has pointed out, Donnan equilibrium effects in ion-exchange columns can appreciably change the pH of a mobile phase that has a low salt concentration. The pH in the matrix of an anion-exchange column will increase because OH^- ions are retained by the exchange sites, while protons increase in the bulk mobile phase. We have observed a two-unit decrease in the pH of a dilute Pi buffer during its passage through an anion-exchange column. Localized changes in the matrix could be quite large and appreciably change nucleotide ionization–protonation and retention. These column-induced pH changes also increase the time required for pH equilibration of the column.

In this paper we describe procedures to accomplish two objectives: (1) separate plant nucleotides from other substances that interfere with their determination by HPLC; (2) improve the control of mobile phase pH so that nucleotide retention times and separations are reproducible and column pH equilibration is reasonably fast.

EXPERIMENTAL

HPLC equipment

The HPLC equipment consisted of the following: Two Altex* Model 100 pumps with a Model 420 microprocessor control (Altex, Berkeley, CA, U.S.A.); Schoeffel Model SF770 variable-wavelength UV detector (Schoeffel Division, Kratos, Westwood, NJ, U.S.A.); Spectra-Physics Model SP 4100 computing integrator (Santa Clara, CA, U.S.A.); Rheodyne Model 7120 injector with a 20-μl loop (Cotati, CA, U.S.A.); Whatman Partisil-10 SAX anion-exchange column (250 × 4.6 mm I.D., Clifton, NJ, U.S.A.); silica saturator pre-column (250 × 4.6 mm I.D., packed with 50-μm silica gel); guard column (55 × 4.6 mm I.D., packed with pellicular anion exchanger, Vydac, Hesperia, CA, U.S.A.). The analytical column and the silica saturator precolumn were kept at 30°C with water jackets. The guard column was at ambient temperature. The pH of the column efflux was monitored with a micro pH flow cell (Markson J-738, Phoenix, AZ, U.S.A.), connected downstream from the UV-detector.

Low-pressure columns

The small, low-pressure columns used to separate nucleotides from the many other compounds and ions present in plant extracts were prepared from glass syringe barrels, fitted with PTFE valves (Bio-Rad, Richmond, CA, U.S.A.), and flanged PTFE tubing connections (Alltech, Deerfield, IL, U.S.A.). The syringe size was 10 ml for XAD-2 and water-insoluble polyvinylpolypyrrolidone (PVP) and 5 ml for charcoal–cellulose. A disc of glass wool, 2–3 mm thick, was placed on the bottom of the syringe and the packing added as an aqueous slurry. The drained bed volume

* Company and trade names are shown for benefit of the reader and do not imply endorsement or preferential treatment by the USDA of the company or products noted.

was 10 ml for XAD-2 and PVP and 3 ml for charcoal–cellulose. The bed surface was protected with a glass wool disc. The three columns were connected in series (Fig. 1) and equilibrated with 0.01 *M* hydrochloric acid before extract was allowed to flow through them.

Before XAD-2 was packed in a column, it was triturated with water in a blender to reduce the particle size. Fine particles that did not settle in 1 min were discarded. Both XAD-2 and PVP were boiled in 3 *M* hydrochloric acid for 1 h and left in the hydrochloric acid over night. They were washed sequentially with water, 1 *M* sodium hydroxide, water, methanol, and boiled in water under reduced pressure to remove the methanol. Used XAD-2 and PVP were regenerated by Soxhlet extraction with methanol.

Charcoal was purified by heating it with an excess of concentrated sulphuric acid at 150°C for 10 h. The mixture was allowed to cool overnight and then slowly poured into a large volume of water. After the charcoal had settled, the supernatant was decanted and discarded. The charcoal was washed with water by suspending, settling, and decanting, until the pH had risen to 5. It was then washed on a Büchner

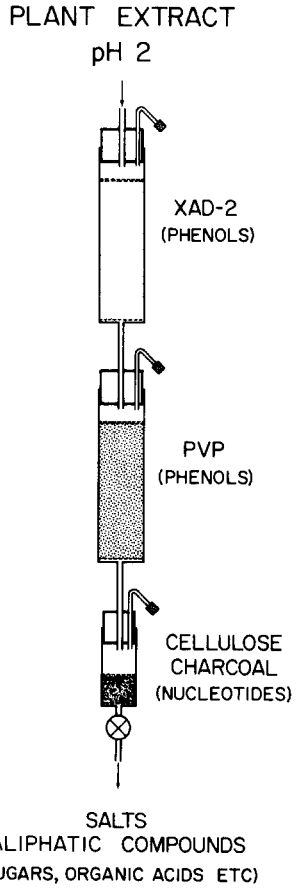

Fig. 1. Three columns used to separate nucleotides in plant extracts from interfering substances. The columns were constructed of glass syringe barrels, 10 ml for XAD-2 and PVP and 5 ml for charcoal.

funnel with methanol, water, and air dried. To prepare a charcoal column, 2 g of charcoal were thoroughly mixed with 6 g of cellulose (Whatman CF 11) in 100 ml of water. The cellulose was added to prevent the fine, friable charcoal particles from settling into an impermeable bed. With the cellulose present, a flow of 1 to 2 ml/min was maintained. The charcoal–cellulose slurry was poured into a 5 ml syringe, with a glass wool pad on the bottom, and allowed to settle by gravity for 2 min. Fine particles still suspended were aspirated off. This process was repeated until the bed volume was 3 ml. The bed surface was protected with a thin glass wool disc. From tests with nucleotide standards we estimate the sorption capacity of this charcoal bed at >6 μmoles of nucleotide per ml. Each ml has enough capacity to sorb all the nucleotides in about 10 g of fresh, mature bean leaves. The sorption capacity tended to decrease with increased ionic strength of the extract.

Materials

Amberlite XAD-2 (20–50 mesh beads) was obtained from Rohm and Haas (Philadelphia, PA, U.S.A.); PVP from Calbiochem-Behring (San Diego, CA, U.S.A.); cellulose powder, CF 11, from Whatman; activated charcoal (USP powder), KH_2PO_4, and acetic acid (analytical reagent grade) from Mallinckrodt (St. Louis, MO, U.S.A.); Pyrex glass wool sheets from Corning Glass (Corning, NY, U.S.A.). Standard nucleotides and sugar-nucleotides were obtained from Sigma (St. Louis, MO, U.S.A.). The laboratory-deionized water was further purified before use by passage through columns of analytical-grade mixed-bed resin (Bio-Rad) and XAD-2. The KH_2PO_4 was purified by Shmukler's[13] procedure. Insoluble material was removed from HPLC solvents and plant extracts by filtering through 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A.).

Plant extract preparation for HPLC

From 5 to 20 g of fresh plant tissue was frozen in liquid nitrogen and ground to a frozen powder in a cold mortar. The powdered tissue was extracted twice by trituration with ice-cold perchloric acid (PCA, 2 ml/g fresh tissue), followed by centrifugation (15 min, 40,000 *g*, 2°C). The first trituration was with 0.5 *M* PCA in a mortar, the second with 0.2 *M* PCA in the centrifuge tube. The two extracts were combined and neutralized with 1 *M* potassium hydroxide. Precipitated $KClO_4$ was removed by centrifugation at 2°C. The extract was frozen, thawed, and centrifuged once more. The supernatant was adjusted to pH 2 with hydrochloric acid and allowed to flow (1–2 ml/min) through the connected XAD-2, PVP, and charcoal columns (Fig. 1). At pH 2, XAD-2 and PVP sorb most of the aromatic compounds in the extract but not the nucleotides[10]. These are sorbed by the charcoal. Inorganic ions and aliphatic compounds pass through all three columns. The extract was washed through the three columns with cold 0.01 *M* hydrochloric acid until the UV absorbance of the effluent at 210 nm, due to sugars, organic acids, etc. was <0.01. The charcoal column was disconnected from the other two and washed with 10 ml of water to remove hydrochloric acid. The nucleotides were eluted with an ethanol–water–ammonium hydroxide solution, 35:65:0.3 (v/v/v) for the first 20 ml, then 65:35:0.3 until elution was complete, usually about 150 ml. Starting with the lower ethanol concentration reduced bubble formation on the charcoal column. Eluents are best prepared a day ahead to allow bubbles to escape. The ethanol eluent was

collected on ice, vacuum distilled at 40°C to remove ammonia and ethanol, and lyophilized. The residue was taken up in a small volume of water and forced through a 0.45-μm membrane filter. This solution was injected into the HPLC column.

Column-induced changes in mobile phase pH

Changes in the mobile phase pH caused by Donnan equilibrium effects in the anion exchange column and the influence of these changes on nucleotide retention times were investigated with both isocratic and gradient elution. We will designate the pH of solution entering the column as the influx pH and the pH of solution leaving the column as the efflux pH. With isocratic elution, the influx pH was constant and the efflux pH was monitored with a pH flow cell. A 4-nucleotide standard was injected into the column. As soon as elution was completed, the standard was again injected. Injection and elution were repeated up to 8 times while solvent was pumped through the column at a constant rate of one ml/min.

During gradient elution, the influx pH changed slightly as the gradient developed. Because our pH flow cell could not tolerate high pressure, we measured the influx pH in separate experiments with the analytical column removed. Before a gradient separation of nucleotides was begun, the starting (A) buffer was pumped through the column for over an hour until the efflux pH was fairly constant though not necessarily equal to the influx pH. A gradient program was initiated and a standard containing 16 nucleotides was injected. The flow-rate was constant at one ml/min. After the elution was completed, the mobile phase was gradually changed from B back to A over a 5-min period. After a reequilibration period with A, the gradient program was begun again and the nucleotide standard reinjected. The gradient elution experiments were conducted with Pi solutions alone and with Pi plus other buffers.

RESULTS AND DISCUSSION

Plant nucleotide purification

Purification of plant nucleotides with the three columns is demonstrated by comparison of the UV-absorption spectrum of an untreated extract of tomato leaves with the spectrum of nucleotides isolated from that extract (Fig. 2). The whole extract had an absorption minimum at about 260 nm and a strong absorbance in the 300–350 nm range, typical of phenols. The nucleotide fraction eluted from the charcoal column had a characteristic nucleotide absorption spectrum with no absorbance above *ca.* 315 nm, indicating the virtual absence of phenols.

Column-induced changes in mobile phase pH

The isocratic elution experiments (Figs. 3 and 4) show the large changes in pH of a dilute Pi mobile phase, caused by its passage through the anion-exchange column, and the concomitant changes in the retention times of the 4 nucleotides. With 0.007 *M* H_3PO_4 at pH 3.5, a little more than one pH unit above its pK_1, about 2.5 h were required for the column to become pH equilibrated (Fig. 3) and for the retention times to stabilize. With 0.005 *M* KH_2PO_4 at its own pH of 4.69, more than 8 h were required (Fig. 4). Clearly, these Pi concentrations were too low to control pH.

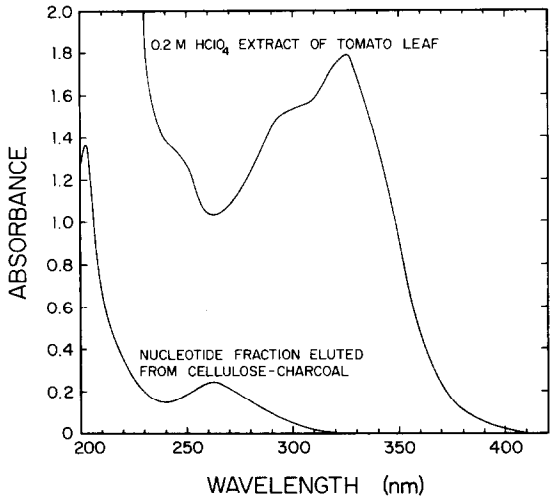

Fig. 2. UV-absorption spectra of the whole perchloric acid extract of a tomato leaf, diluted 1:10, and of the nucleotides in that extract that were eluted from the charcoal column.

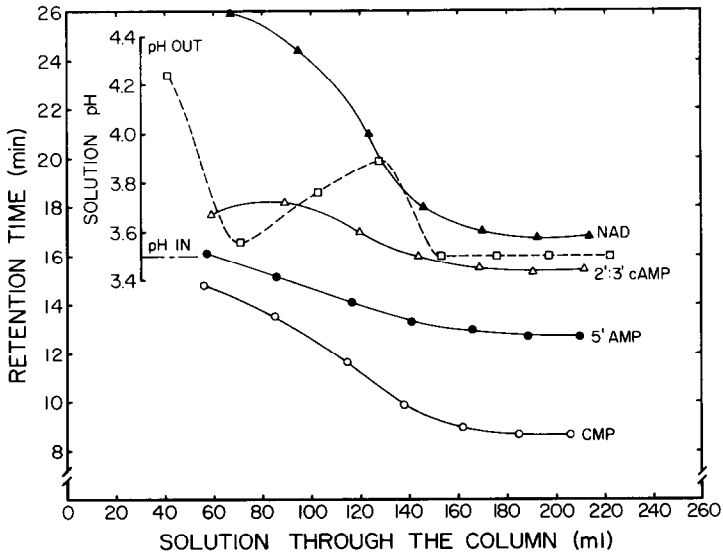

Fig. 3. Changes in the pH of a 0.007 *M* H_3PO_4 mobile phase, caused by its passage through the analytical anion-exchange column and the consequent changes in the retention times of NAD, 2':3'-cAMP, 5'-AMP, and CMP. The H_3PO_4, at an initial pH of 3.5, was pumped through the column at 1 ml/min. The efflux pH was monitored. After 1 h equilibration, a standard containing the 4 nucleotides was injected and the retention times were determined. The injection was repeated at intervals of about 30 min. About 2.5 h were required for column pH equilibration.

The column also changed the pH of the mobile phase during a gradient elution that began with a low Pi concentration (Figs. 5 and 6). In the first example (Fig. 5) the A buffer (0.005 *M* KH_2PO_4 at its own pH of 4.69) was pumped through the column for 4 h before the gradient program was begun and the nucleotide standard

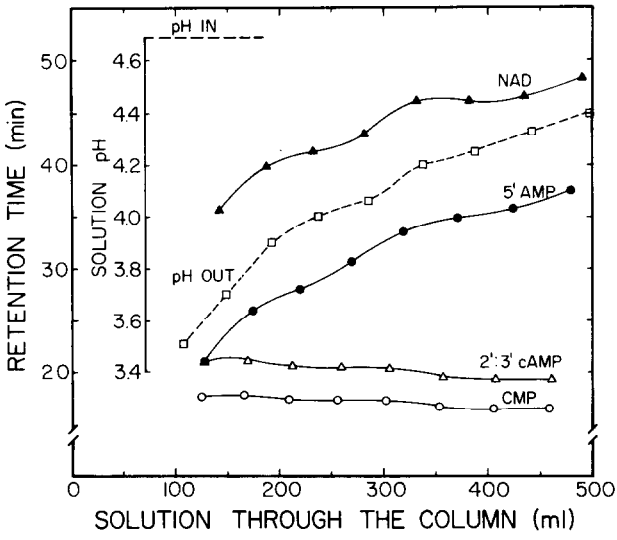

Fig. 4. Changes in the pH of a 0.005 *M* KH_2PO_4 mobile phase caused by its passage through the analytical anion-exchange column and the consequent changes in the retention times of the 4 nucleotides. The KH_2PO_4 at an initial pH of 4.69 was pumped through the column at 1 ml/min. The efflux pH was monitored. After 1.8 h equilibration, the 4-nucleotide standard was injected and the retention times were determined. The injection was repeated as soon as NAD had been eluted. The efflux pH and the retention times were still changing after more than 8 h of pumping.

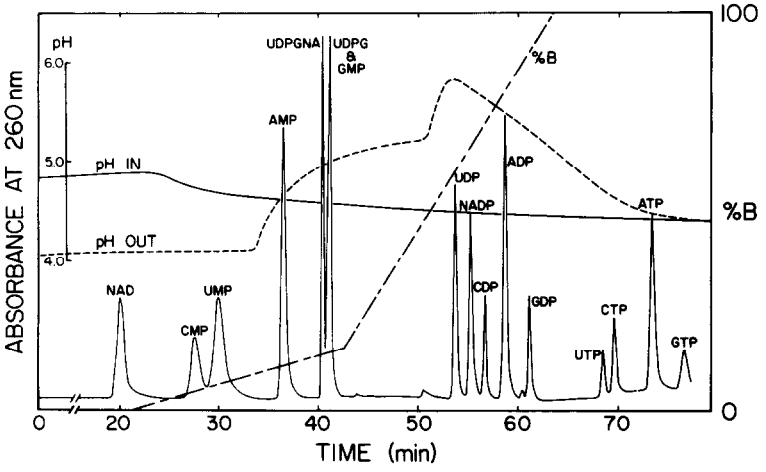

Fig. 5. Chromatogram of nucleotide standards, separated by a Pi gradient. A buffer (0.005 *M* KH_2PO_4, pH 4.69) was pumped through the column at one ml/min for 4 h before the standard was injected and the elution program begun at time 0. Even so, the column was not pH-equilibrated; the efflux pH (dashed line) was fairly stable, but well below the influx pH (solid line). The elution program, indicated by the dots and dashes, was as follows: 0 to 21 min, A only; 21 to 42.5 min, linear increase in B (0.6 *M* KH_2PO_4, pH 4.46) from 0 to 16%; 42.5 to 63.5 min, linear increase in B from 16 to 100%; 63.5 to 77.5 min, 100% B. UDPG = uridine-5'-diphosphoglucose; UDPGNA = uridine-5'-diphospho-N-acetyl-glucosamine. The rest are standard nucleotide symbols.

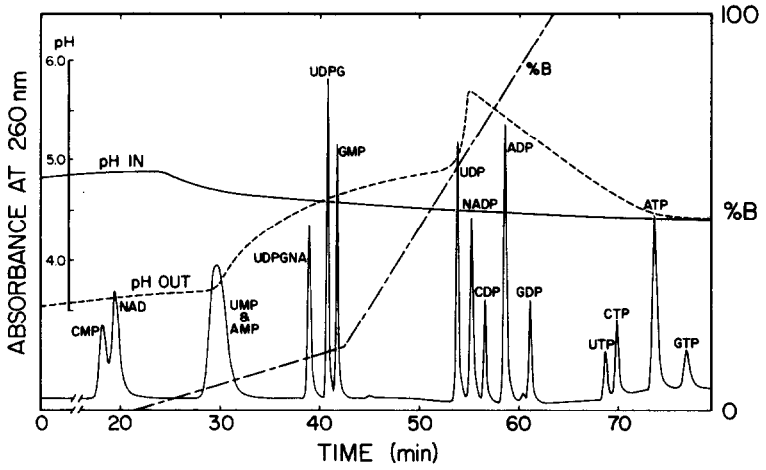

Fig. 6. A second separation of the nucleotide standards by the same buffers and elution program as used in Fig. 5. After that separation, the mobile phase was changed back to A and pumped through the column for 1.5 h. The column was not pH-equilibrated in that time, but an elution program was begun and the standard was injected.

was injected. The efflux pH was fairly stable when the standard was injected but it was still considerably below the influx pH. The B buffer in all of the gradient elution experiments was 0.6 *M* KH_2PO_4 at its own pH of 4.5. The pH was measured on a 1:10 diluted aliquot to avoid the electrode artifact caused by high salt concentration. The influx pH decreased slightly as the Pi gradient began. The efflux pH, on the other hand, increased sharply after a lag of about 12 min. It began to level off, and then abruptly increased again following the increase in slope of the gradient. The efflux pH reached a maximum more than a full pH unit above the influx pH, then decreased till it equalled the influx pH. These results are about as expected from Donnan equilibrium considerations. With a dilute Pi mobile phase, exchange sites retain OH^- ions and allow protons to increase in the bulk mobile phase. When Pi is increased at a pH of about 4.5, $H_2PO_4^-$ eventually becomes the dominant anion in both the exchange matrix and the bulk mobile phase. As a result, the zeta potential, between ions sorbed on a surface and ions in bulk solution, is diminished and Donnan equilibrium effects become less important. Eventually the Pi concentration in the bulk phase is sufficient to control pH and elution.

With the exception of UDPG and GMP, which were eluted as one peak, the nucleotides in Fig. 5 were reasonably well separated, especially the di- and triphosphates that are eluted only at the higher Pi concentrations. After completion of the chromatogram (Fig. 5), the mobile phase was changed back to A and this was pumped through the column for 1.5 h, not long enough for column pH equilibration. The efflux pH was still more than a full pH unit below the influx pH. Nevertheless, a gradient program was initiated and the nucleotide standard was injected. The chromatogram is shown in Fig. 6. The retention times of the first seven peaks were considerably changed from those of the previous chromatogram (Fig. 5). Even the elution order of CMP and NAD had changed, and the UMP and AMP peaks were completely fused. On the other hand, uridine-5′-diphospho-N-acetyl-glucosamine

(UDPGNA), UDPG, and GMP were better separated than on the first chromatogram. As before, the di- and triphosphates were well separated and their retention times were similar on the two chromatograms. The pH tracings for the last 25 min also were similar for the two chromatograms.

Clearly, better control of the mobile phase pH is needed during the first 45–50 min of the chromatogram. After that, the Pi concentration became high enough to control the elution. Both acetate[14] and citrate[15] have been used with dilute Pi to improve the control of pH. We have tried both. The minimum effective concentration for pH control was about 0.01 *M*. Acetate at that concentration showed remarkably little tendency to compete with Pi for anion-exchange sites on the column and, hence, to elute nucleotides. Citrate, on the other hand was an effective competitor. At 0.01 *M* it alone rapidly eluted nucleoside monophosphates and NAD from the column. In our experience the combination of Pi and acetate gave better pH control and better, more reproducible, separations than Pi alone or Pi with citrate. Examples of separations obtained with the phosphate-acetate combination are shown in Figs. 7 and 8. With an A buffer containing 0.005 *M* KH_2PO_4 in 0.01 *M* acetate, adjusted to pH 4.75 with potassium hydroxide, the column was pH-equilibrated (efflux pH = influx pH) within 50 min. The B buffer, as before, was 0.6 *M* KH_2PO_4, pH 4.5. This buffer combination gave good separation of all 16 nucleotides in the standard (Fig. 7). When the chromatogram was completed, the mobile phase was gradually changed back to A. Column pH equilibration was completed within 50 min. The gradient program was reinitiated and the nucleotide standard was injected. The second chromatogram (Fig. 8) closely duplicated the first (Fig. 7). Presumably, the agreement was possible because in both cases the column was at pH equilibrium when the standard was injected and, judging from the efflux pH, it remained in pH equilibrium for the first 25 min. The efflux pH subsequently rose above influx pH as Pi increased

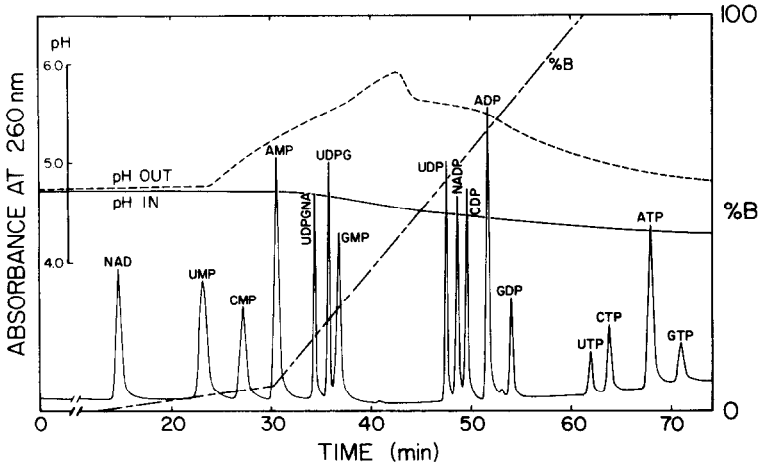

Fig. 7. Chromatogram of nucleotide standards, separated by a Pi–acetate gradient. The A buffer (0.005 *M* KH_2PO_4 in 0.01 *M* potassium acetate, pH 4.75) was pumped through the column at 1 ml/min for 50 min before the standard was injected and the elution program was begun. By then, the column was pH-equilibrated. The elution program was as follows: 0 to 12 min, A only; 12 to 30 min, linear increase in B (0.6 *M* KH_2PO_4, pH 4.46) from 0 to 6.5%; 30 to 61 min, linear increase in B from 6.5 to 100%; 61 to 75 min, 100% B. The symbols are the same as in Fig. 5.

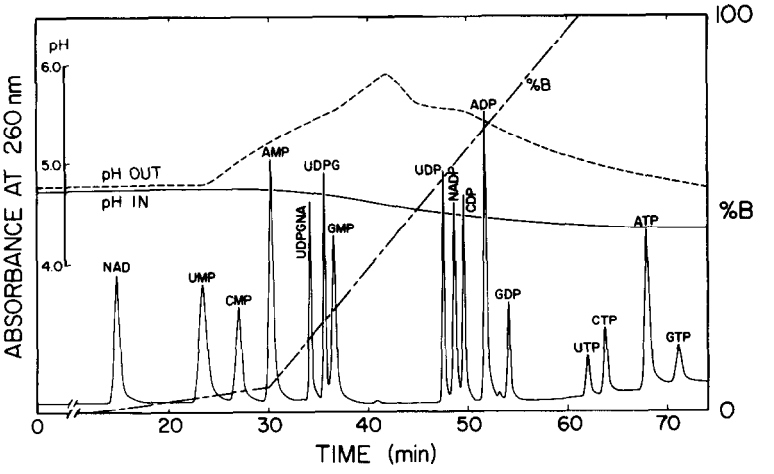

Fig. 8. A second separation of the nucleotide standards by the same buffers and elution program as used in Fig. 7. After that separation, the mobile phase was changed back to A and pumped through the column for 50 min, when the column was pH equilibrated. The elution program was begun and a standard was injected.

and displaced OH^- and acetate ions from exchange sites. But the pH changes were smaller, more gradual, and more reproducible than they were without acetate (compare Figs. 7 and 8 with 5 and 6). It seems especially critical to control mobile phase pH during the early stages of the chromatogram when Pi concentration is low.

Mature photosynthesizing leaves of bush bean (*Phaseolus vulgaris* L.) are a good object for testing procedures for the separation and measurement of nucleotides, because they have low concentrations of nucleotides and relatively high concentrations of interfering compounds and ions. An extract of bean leaves, harvested at mid-day, was prepared and treated with XAD-2, PVP, and charcoal, as described above. The purified and concentrated nucleotide extract was injected into the analytical column and the nucleotides were chromatographed with the same buffers and gradient program as used for the standards in Figs. 7 and 8. The bean-leaf chromatogram (Fig. 9) was generally similar to the standards. The major differences were

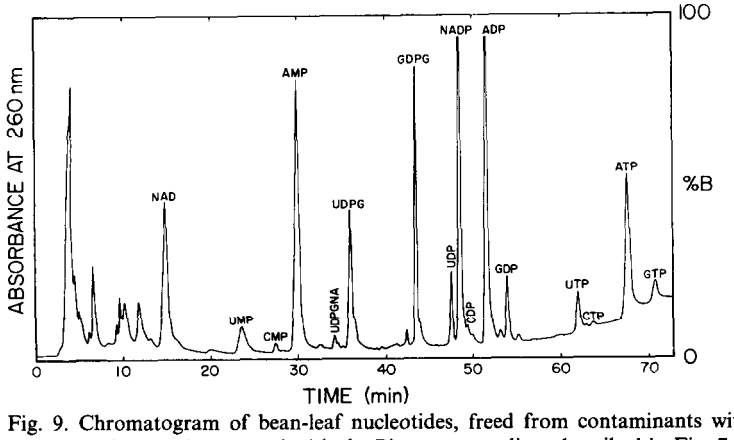

Fig. 9. Chromatogram of bean-leaf nucleotides, freed from contaminants with the tri-column system shown in Fig. 1 and separated with the Pi–acetate gradient described in Fig. 7.

the compounds eluted before NAD, possibly nucleo-bases and nucleosides, and the guanosine-5'-diphosphoglucose (GDPG) peak. As an example of the nucleotide concentration and variability in the leaves, the mean ATP concentration and standard deviation for 9 mature leaves harvested at mid-day was 49 ± 6 nmoles/g of fresh leaf. The ATP concentration was about double this, and AMP nearly disappeared in leaves harvested at midnight. The quality of the bean-leaf chromatogram, we believe, demonstrate the utility of the procedures described for measurement of plant nucleotides.

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