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Analytical Techniques for Cell Fractions. III. Nucleotides and Related Compounds

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INTRODUCTION

The methods described in this paper are devised for the separation of the common nucleotides, nucleosides, and purine and pyrimidine bases on one column at one pH with the compounds quantitated by a sensitive automatic technique.

Following the classical work of Cohn and co-workers (1–6), nucleotides have been generally separated on anion-exchange columns at acid pH's, nucleosides on anion-exchange columns at alkaline pH's, and purine and pyrimidine bases either on anion exchangers or on cation-exchange columns in the acid range (1–7). Separation, though incomplete, of mixtures of bases, nucleosides, and nucleotides on a single anion-exchange column (2) has been achieved by using buffers ranging from pH 10.2 to 2.75. The observation that the pyrimidine nucleotides were much less strongly adsorbed, per unit charge on the molecule, than the purine nucleotides (2) suggested that both nucleotide solubility and interaction with the resin matrix through nonionic forces (in addition to the expected ionic effects) may be responsible for the elution patterns observed. The possibility was considered, therefore, that bases, nucleosides, and nucleotides might be eluted in the same relative order from the same column, providing the proper conditions could be found.

Brief reports of this work have appeared elsewhere (8, 9).

EXPERIMENTAL

It is not feasible to explore systematically all the possible combinations of ion-exchange resin composition, bead size, temperature, flow rate,

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pH, ionic composition of the eluting fluid, elution gradients, and other factors to arrive at an optimal system for the analysis of a given mixture. Thus the problem of separating nucleotides, nucleosides, and bases has been approached in the following manner.

Choice of pH. Systems employing a pH gradient (10) have not been used here because the molar absorptivity and the ratio of absorbances at two wavelengths such as 260 and 280 mμ are pH dependent. If a pH gradient is used, the pH of the solution comprising a given peak must be known. If a compound is not always eluted at exactly the same pH, then the spectral properties of the compounds studied must be available over a range of pH's. The alternate possibility is to adjust the effluent by adding a strong buffer, acid, or base to give a known pH. The latter method is often used in manual analyses. Neither of these methods is desirable, however, in an automatic recording system. The addition of a pH-adjusting solution to the column effluent stream introduces the requirements of constant and known flow rates for both streams.

A further disadvantage of a pH gradient is that the elution order is also pH dependent. In tissue extracts, mono-, di-, and triphosphates thus may not occur in any exactly repeating sequence with a pH gradient. For these reasons the possibility of elution at a single pH has been explored.

The choice of pH is dictated by the sensitivity of purine deoxyribonucleosides and nucleotides to low pH, and by the shift in the position of uridylic acid with respect to other nucleotides eluted from Dowex 1 occurring between pH 2 and 5. The choice of pH in this instance was decided by the behavior of nucleosides and bases on the column. In previous studies (8, 9) promising nucleoside, base, and nucleotide separations were obtained at pH 4.4 with sodium acetate buffers ranging from 0.60–3.0 *M*. A modification of this procedure has been adopted in the present study.

Choice of Resin Cross Linkage. Resins having a low cross linkage (low divinylbenzene content) such as Dowex 1-X8 or Dowex 50-X2 leak appreciable amounts of ultraviolet-absorbing material. With higher cross linkages proportionately less leakage is observed. While such leakage may cause little practical difference with short columns, serious difficulties are experienced with long (150-cm) columns. Dowex 1-X8 was found sufficiently stable for use in long columns at 40°C and did not swell and shrink as much as the 2% cross-linked resin. These studies, therefore, have been restricted to the 8% cross-linked material.

Preparation of Columns. Dowex 1-X8 resin (Bio-Rad) 200–400 mesh, Control No. 4764-30 B-597, was hydraulically fractionated using tap water (11). The fraction chosen for use in the long columns (0.9 × 160

cm) averaged 60 μ in diameter. Resin particle size measurements were made on photographic enlargements of phase contrast micrographs or by direct measurement using an ocular micrometer. When attempts were made to fractionate "through 400 mesh" resin hydraulically, poor fractionation was obtained because very small resin beads adhered tightly to the larger particles. Slight improvement was obtained by carrying out the hydraulic fractionation in dilute HCl.

Before use the resin was treated with normal HCl, NaOH, and acetic acid. The suspension was then stirred in 3 M acetic acid to ensure removal of CO_2 bubbles before packing the column. The settled resin was then suspended in 0.15 M sodium acetate buffer, pH 4.4, for packing. The resin was packed in segments under pressure from a piston pump with a flow rate of approximately 1 ml/min. When the resin had settled, the supernatant fluid was removed by means of polyethylene tubing attached to a suction flask and vacuum line. The column was again filled with resin slurry and the packing procedure repeated until a column 150–160 cm in length was obtained. After packing, the column was washed for several hours with 0.15 M sodium acetate at pH 4.4.

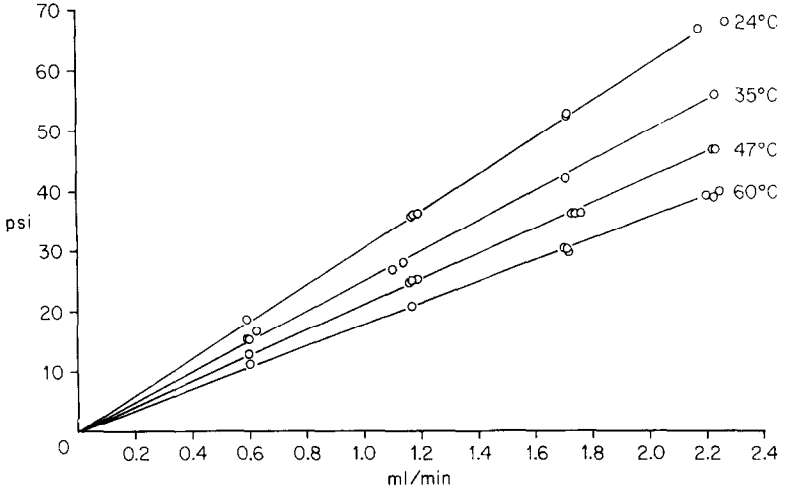

FIG. 1. Flow rates through a 0.9 × 150 cm colmn of Dowex 1-X8, 60 μ fraction (average), as a function of pressure and temperature.

The flow rate is directly proportional to pressure (Fig. 1) and is inversely proportional to the temperature as expected (11) if the resin particles are not deformed with the pressures used.

Compared with the columns used for amino acid analysis, these col-

umns have a fairly low resistance, but are useful where high flow rates are to be studied.

Production of Gradients. Three 2000-ml graduated cylinders were connected in series, with a magnetic stirrer in the cylinder connected to the pump. When two cylinders were used a linear gradient was obtained, whereas three cylinders gave a convex gradient (12). For the long column work the first cylinder contained 600 ml 0.15 *M* sodium acetate and the second (and third if used) contained 3.0 *M* sodium acetate, both at pH 4.4. For the medium column work only two cylinders were used. A 150-watt lamp over the first cylinder warmed the buffer slightly, thereby reducing the level of dissolved gases and preventing the formation of air bubbles in the spectrophotometer cuvette. Other gradient systems applicable to specific mixtures will be described in subsequent papers.

Regeneration of Columns. Regeneration of the resin in the column with 3 *M* sodium acetate and sodium hydroxide has been explored. These procedures shrink the resin bed and increase the back-pressure during the subsequent analysis. It has been found easier and more convenient to use freshly packed columns for each analysis.

Columns are unloaded by clamping them in an inverted position over a large, sintered-glass funnel and washing the resin out by running PE 190 polyethylene tubing up the column. The resin is then regenerated with 1 *N* HCl, 1 *N* NaOH, water, 3 *M* sodium acetate pH 4.4, followed by 0.15 *M* sodium acetate pH 4.4. The resin is then repacked in the column. It is convenient to have one or two extra long columns packed at any given time.

Chromatographic Systems. While previous studies (9) showed that nucleosides, bases, and nucleotides could be separated at pH 4.4 in sodium acetate, considerable variation in the behavior of different batches of resin with respect to the separation of uridine and uracil, thymine and inosine, adenosine and adenine, and guanosine and guanine have been observed. A fraction obtained from "through 400-mesh" resin achieved better separation at 20°C, whereas the resin used in the present studies was better for this separation at 40°. All work described here has been done on a single batch of resin, Control No. 4764-30 B597 obtained from Bio-Rad Laboratories.

Since these investigations are also preliminary to studies on tissue acid-soluble nucleotides, thymine and inosine have also been incorporated in the test mixtures examined.

Standards.[5] It is convenient to make up 10 m*M* single solutions of each

[5] Abbreviations in this paper are based on accepted usage of the *Journal of Biological Chemistry,* as follows:
Bases: Cyt, Ura, Thy, Ade, Gua.

compound in distilled water with the exception of guanine, which is made up to 1.0 m*M*. From these stocks the following standard mixtures are prepared to be 2 m*M* with respect to each compound, except guanine, which is present in a final concentration of 0.2 m*M* and 5-methyl-CMP in a final concentration of 0.5 m*M*.

STANDARD MIXTURES

Standard	Mixture
A	Cyt, Cyd, Urd, Ura, Thy
B	Ino, Ado, Ade, Guo, and Gua (0.2 m*M*)
C	2′(3′)-CMP, -UMP, -AMP, and -GMP
D	CMP, UMP, IMP, AMP, and GMP
E	dCyd, dAdo, dGuo, and Thd
F	dCMP, dTMP, dAMP, dGMP plus 5-methyl-dCMP (0.5 m*M*)
G	Cyt, Thy, Ade, Gua (0.2 m*M*)
H	CDP, UDP, ADP, and GDP
I	CTP, UTP, ITP, ATP, and GTP

RESULTS

The separation of the initial group—cytosine, cytidine, uridine, uracil, thymine, and inosine—was studied in detail. The separations achieved with the batch of resin used as a function of temperature and buffer concentration are shown in Fig. 2. On the basis of these observations we chose a temperature of 40°C. The peaks in the first group were very sharp and would be incompletely distinguished in manual analysis unless fractions of less than 2 ml were collected. To make the integration easier, peaks of the first group of compounds were expanded by using a slower flow rate, until after the inosine peak had been eluted. (This pump rate change may be delayed until after adenosine if the adenosine peak is too sharp.) The pump rate changing mechanism has been described (13). The complete separation of the major components of RNA, using a linear gradient of 1400 ml total volume extending from 0.15 to 3.0 *M* (with respect to acetate) sodium acetate, pH 4.4, is shown in Fig. 3b. The relative absence of 2′-uridylic acid in the standard mixture is ex-

Ribosides: Cyd, Urd, Ino, Ado, Guo.
Ribonucleotide monophosphates: [2′(3′)- if indicated, otherwise 5′] CMP, UMP, IMP, AMP, GMP.
Ribonucleotide diphosphates: CDP, UDP, IDP, ADP, GDP.
Ribonucleotide triphosphates: CTP, UTP, ITP, ATP, GTP.
Deoxyribosides: dCyd, dAdo, dGuo, Thyd.
Deoxyribonucleotides: dCMP, dTMP, dAMP, dGMP, 5-methyl-dCMP.

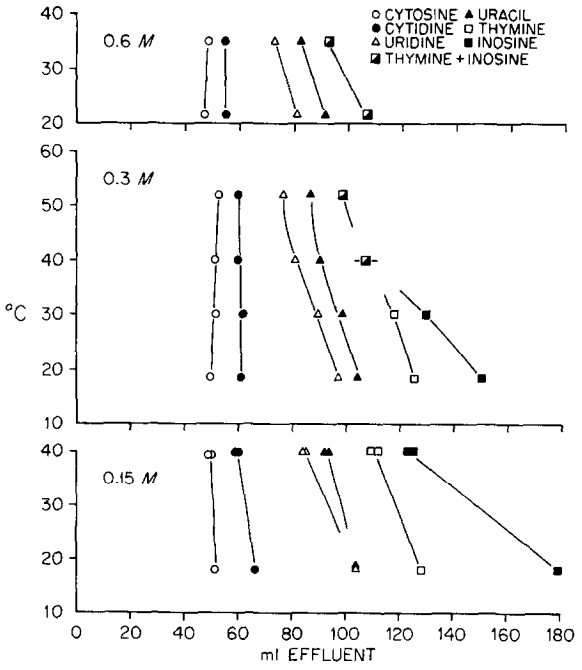

FIG. 2. Separation of mixture of bases and ribonucleosides as a function of temperature using Dowex 1-X8, 60 μ fraction, in a 0.9 $\times$ 150 cm column, and three concentrations of sodium acetate at pH 4.4.

plained by the failure of the 2′ isomer to crystallize readily. The commercial samples marked as mixed 2′, 3′ isomers, therefore, were almost entirely the 3′ isomer.

Variation in sample volume has been found to affect the initial separations, especially the cytosine-cytidine pair. Sample volumes, therefore, were kept to 0.5–1.5 ml. The pH of the sample was adjusted with 3.0 M sodium acetate to approximately pH 4.4.

To show the position of the 2′-uridylic peak, a commercial sample of yeast RNA was hydrolyzed for 18 hr at 37°C in 0.15 N KOH, neutralized, concentrated, acidified to pH 4.4, and chromatographed as described for the known mixture. The results, shown in Fig. 3, reveal the lower limits of the method. It is apparent that adequate separation of the 2′- and 3′-UMP is achieved.

The application of the same system to a mixture of bases, deoxyribonucleosides, and deoxyribonucleotides is shown in Fig. 4.

Tissue acid-soluble nucleotides and their derivatives constitute a very complex mixture whose complete resolution by any one system is unlikely. As a first approach to this problem we have examined the separa-

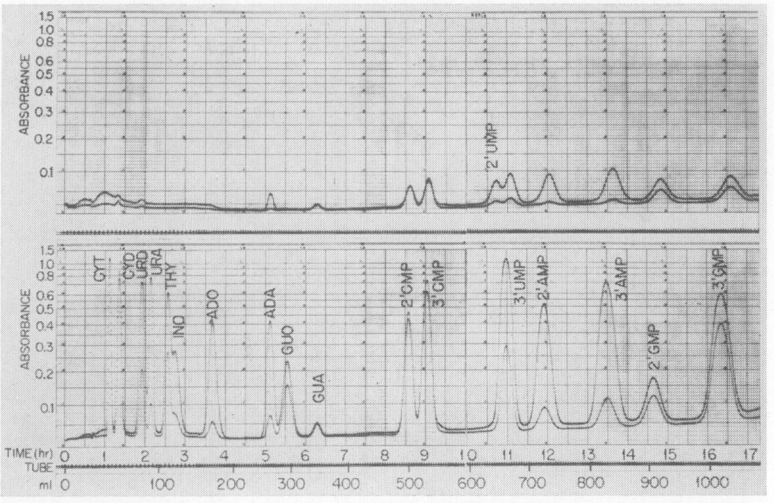

FIG. 3. (a) Top: chromatogram of an alkaline hydrolyzate of 0.5 mg yeast RNA (Pabst). Absorbance recorded at 260 and 280 mμ using a 1-cm quartz flow cell. A convex gradient of 600 ml 0.15 *M* sodium acetate, pH 4.4, grading into 1200 ml 3 *M* sodium acetate at pH 4.4 was employed. (b) Bottom: separation of a synthetic mixture of bases, nucleosides, and ribonucleotides using same procedure as in (a). Sample (1.4 ml total) contained 0.2 ml each of standards A and B, and 1.0 ml of C. Pump rate changes from 30 to 50% stroke at 3¼ hr (after 140 ml). Note that 2′-UMP is missing from the standard; 4.13 ml per siphon spill. Temperature, 40°C throughout.

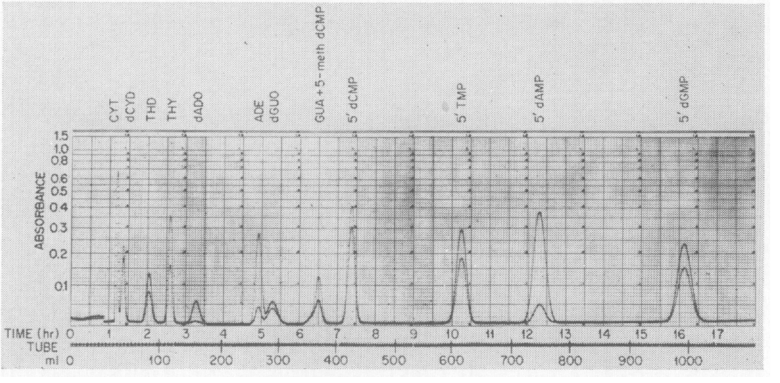

FIG. 4. Separation of mixture of purine and pyrimidine bases, deoxyribonucleosides, and deoxyribonucleotides. Dowex 1-X8 column, 0.9 × 155 cm. Flow rate changed from 0.715 to 1.10 ml/min at 2¾ hr. Absorbance recorded with 0.2-cm cell. Sample: 0.4 ml standard G, 0.2 ml E, 1.5 ml F.

tion achieved with more complex mixtures of known substances including naturally occurring di- and triphosphates. The results are shown in Fig. 5. Baseline compensation was not used so as to show the extent of

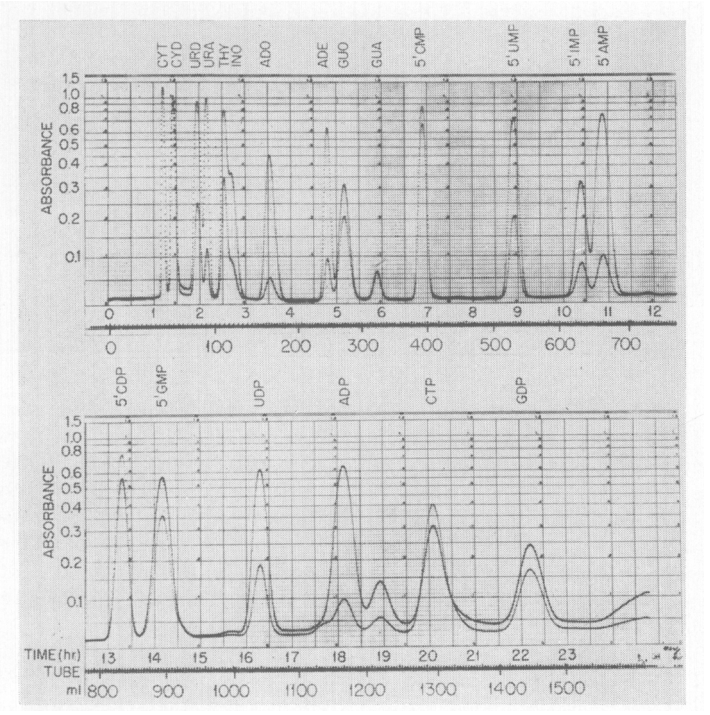

FIG. 5. Separation of a mixture of 5'-nucleotides, ribonucleosides, and bases. Chromatographic conditions identical to those described for Fig. 4. Sample: 0.2 ml standard A, 0.2 ml B, 0.5 ml D, 0.5 ml H, and 1 ml I. 1-cm light path, quartz, flow cell used.

the rise in absorbancy caused by the elution gradient. This method is a modification of that used for monophosphates in that 3 *M* sodium acetate was pumped through the column after completion of the gradient. The method is successful through the elution of UTP. ATP and GTP invariably showed tailing.

Separation of Triphosphates on a Medium Column. To eliminate the lengthy terminal portion of the run necessary to elute UTP, ATP, and GTP, a number of faster methods involving shorter columns were explored. The most successful of these utilizes a 1-liter gradient at pH 3.6 as shown in Fig. 6. The more concentrated buffer contains 1.0 *M* Ac^-, 0.5 *M* Cl^-, with Na^+ to pH 3.6. The gradient was a linear one from a 50% dilution with distilled water of the above to the stock solution. The

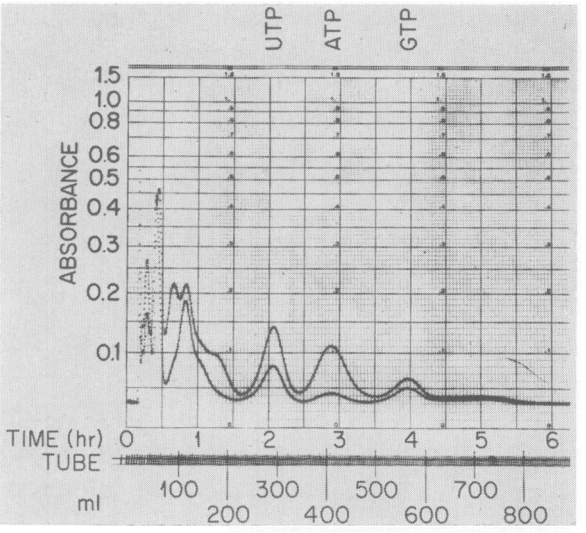

FIG. 6. Medium column separation of UTP, ATP, and GTP. Dowex 1-X8 resin, 0.9 × 100 cm column operated at room temperature with a flow rate of 2.4 ml/min. Elution gradient: 500 ml 1:1 dilution of stock buffer grading linearly into 500 ml undiluted buffer. Stock buffer contains 1.0 *M* Ac^-, 0.5 *M* Cl^-, with Na^+ to pH 3.6.

column was 50 × 0.9 cm and was operated at room temperature, using a flow rate of 1.7 ml/min. This analysis may be completed in 6 hr.

Considerable effort was expended to shorten the time for the triphosphate analysis by using higher column temperatures. At 40° very little breakdown of ATP to either AMP or ADP was observed either when the column was eluted at once after loading or when the sample was left on the column at 40° for 4–10 hr before elution was started. However, a very skewed peak was always observed after ATP. Its pattern was that expected of a more acid substance constantly being formed on the column during elution. Since this substance has a higher affinity for the resin than ATP, it appears probable that no phosphate has been lost. The lack of change in the 260/280 ratio suggests that little change has occurred in the adenine moiety. Since a small amount of the trailing peak is seen during elution at room temperature under conditions which are not extremely acid (Fig. 6), it is probable that the alteration may be common to most procedures for chromatographing ATP on ion-exchange resins. Further investigation of this effect is in progress.

Mathematical Analysis of Curves. While Gaussian curves may be expected when single substances are eluted from ion-exchange columns with a solution of constant composition, some skewing of the curves may be expected when gradient elution is employed (2). It is of interest, there-

fore, to know whether the departures actually observed with the systems here are significant. All peaks in the chromatograms shown in this paper were analyzed by replotting the data on Gaussion chart paper (14). An example is shown in Figs. 7 and 8. No appreciable departures from nor-

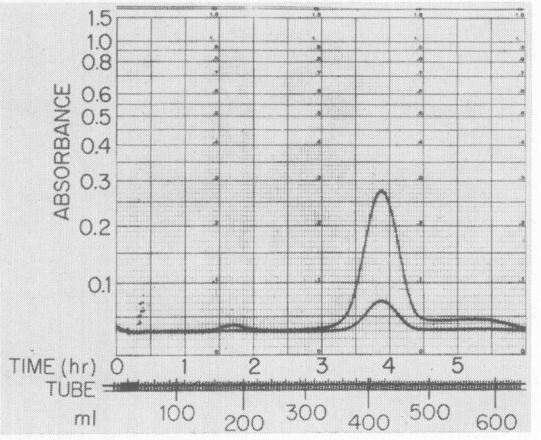

FIG. 7. Chromatography of 5 μM ATP under conditions identical to those used in Fig. 6. Note shape of tailing observed after ATP peak.

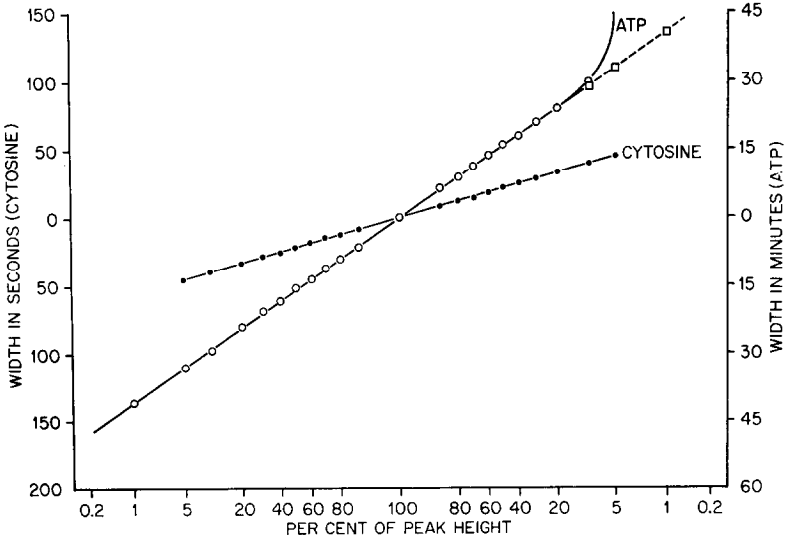

FIG. 8. Plot of ATP curve shown in Fig. 7, and cytosine peak from Fig. 3b on Gaussian chart paper. Gaussian curves are thus obtained at both ends of the gradient. Extrapolated points on the ATP curve were used to plot rest of curve shown in Fig. 7.

mal curves were seen except where two peaks overlapped, with adenosine which often exhibits slight tailing, and ATP and GTP. It appears, then, that quantitative results may be obtained by the simple integration method previously described (13).

When two curves overlap so that the toe of one produces a negligible effect on the peak height of the other, the outer portions of each curve may be inflected back to reconstruct the overlapping portions. If one curve is much larger than the other, a point subtraction may be made which should leave a normal-shaped minor curve, as is shown in Figs. 9 and 10. Peak overlap produces little effect when the value of the width at

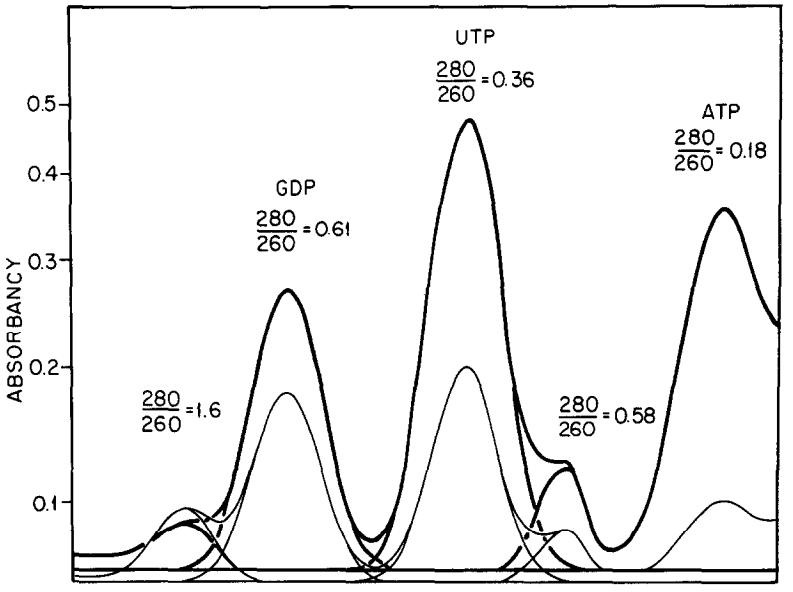

FIG. 9. Analysis of overlapping curves. GDP and UTP curves from a run similar to that shown in Fig. 5 but with a mixture containing unknown pre-GDP and post-UTP peaks. The 260 and 280 mμ GDP and UTP curves were plotted on Gaussian paper and the toe portions of both curves constructed from the extrapolation of the Gaussian plots. By subtracting the constructed GDP and UTP curves from the pre-GDP and post-UTP curves, the latter were isolated, and their 280/260 ratios determined.

half-height is not affected, such as with ATP separated by the short column method described here.

It is evident that the systematic analysis of the shapes of curves obtained with tissue extracts, for example, may yield information on many minor compounds hidden beneath major peaks, and that the data may be adapted to computer analysis (15).

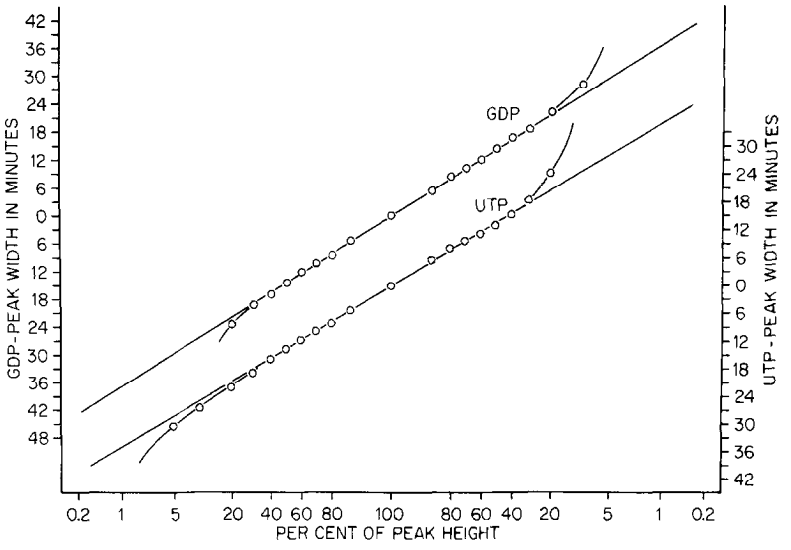

FIG. 10. Plot of 260 mμ absorption curves from Fig. 9 on Gaussian paper. Extrapolations plotted back on curves in Fig. 9 and used to compute values for the pre-GDP and post-UTP curves.

Quantitation of Results. The peak areas may be obtained as previously described (13) and the amount of a given ultraviolet-absorbing substance readily calculated. Spectrophotometric data for a number of compounds at pH 4.4 and 3.6 are listed in Table 1. The values were determined by multiplying the published values by experimentally determined ratios of the absorbancy at pH 4.4 (or 3.6) and at one of the pH's used in the published work. The 280/260 ratios measured on peaks separated here are also given.

Studies with Uncharged Resin Particles. The suggestion that the purine or pyrimidine moiety of the nucleotide molecule may interact with the resin particle through other than ionic forces was the starting point of the present work, which has led to the successful separation of bases, nucleosides, and nucleotides on the same column at a single pH. It appeared of interest, therefore, to explore chromatography of these compounds on the uncharged resin particle to see whether interaction with the resin matrix could be demonstrated.

Eight per cent cross-linked resin particles, 200–400 mesh, identical to those used in the preparation of Dowex 1 but lacking charged groups were washed free of fines with distilled water. The resin was packed to give a 160×0.9 cm column, and was equilibrated for several hours with 0.15 M sodium acetate at pH 4.4. A mixture of bases, nucleosides, and

TABLE 1

SPECTROPHOTOMETRIC DATA ON COMMON BASES, NUCLEOSIDES, AND NUCLEOTIDES SYSTEMS DESCRIBED IN TEXT

Compound[a]	pH 4.4				pH 3.6				Reference Data			
	$\alpha_M \times 10^{-3}$ 260	280	Calc.[b] 280/260	Obs.[c] 280/260	$\alpha_M \times 10^{-3}$ 260	280	Calc.[b] 280/260	Obs.[c] 280/260	$\alpha_M \times 10^{-3}$	λ	pH	Reference
Ade (C)	13.2	2.82	0.213	0.22	-	-	-	-	13.1	262	1	(16)
Ado (P)	15.2	2.52	0.169	0.15	-	-	-	-	15.4	259	7	(16)
2′ AMP ( )					-	-	-	-	15.0	260	7	(17)
3′ AMP ( )				0.174	-	-	-	-	15.0	260	7	(17)
5′ AMP (P)	15.1	2.60	0.172	0.174	-	-	-	-	15.4	259	7	(16)
ADP (P)	15.2	2.85	0.187	0.	15.15	3.17	0.21	0.	15.4	259	7	(16)
ATP (P)	15.4	2.59	0.168	-	15.0	3.12	0.21	0.	15.4	259	7	(16)
dAdo (P)	14.6	2.24	0.153						14.8	259	7	d
Cyt (C)	6.12	7.42	1.21	1.21	-	-	-	-	10.0	276	1	(17, 18)
Cyd (C)	7.0	9.1	1.3	1.32	-	-	-	-	13.0	280	2	(19)
2′ CMP	-	-	-	1.37	-	-	-	-	7.6	260	7	(17)
3′ CMP				1.43	-	-	-	-	7.6	260	7	(17)
5′ CMP (P)	6.62	10.3	1.56	1.53	-	-	-	-	13.0	280	2	(16)
CDP (P)	6.29	11.0	1.75	1.	6.5	11.8	1.82	.	13.0	280	2	(16)
CTP (P)	6.2	10.7	1.72	1.	6.5	12.1	1.88	.	13.0	280	2	(16)
dCyd (C)	6.88	9.81	1.42	-	.	.	.		12.95	280	2	d

(table cont'd.)

mono-, di-, and triphosphates was placed on the column and eluted with 0.15 *N* sodium acetate at pH 4.4 at 40°C. More than 99% of the added ultraviolet-absorbing material was recovered in four tubes (16 ml) after approximately one column volume (Fig. 11). No evidence of interaction with the uncharged resin particles was noted.

Calibration of Wavelength Settings of the DB Spectrophotometer. Small changes in the wavelength settings produce large changes in the absorbance measurements when the wavelength used is not close to the absorption maximum. The instruments employed in the present study have been in use over a year, and it gradually became evident that the expected ratios (especially for the cytidylic acid peaks) were not being observed. It appeared that the wavelength-shifting mechanism may have caused some wear in the cam system that moves the prism. Since this wear may be localized, calibration at points other than 260 and 280 mμ would be of little help. It is important, therefore, to have available a simple method for calibrating the 260 and 280 positions.

TABLE 1 (Cont'd.)

Compound [a]	pH 4.4				pH 3.6				Reference Data			
	$\alpha_M \times 10^{-3}$ 260	280	Calc. [b] 280/260	Obs. [c] 280/260	$\alpha_M \times 10^{-3}$ 260	280	Calc. [b] 280/260	Obs. [c] 280/260	$\alpha_M \times 10^{-3}$	λ	pH	Reference
Gua (C)	6.1	6.0	0.98	1.02	-	-	-	-	11.1	249	1	(20, 21
Guo (C)	11.8	8.0	0.68	0.65	-	-	-	-	13.7	252	7	(19, 22
2′ GMP	11.8	8.0	0.68	0.67	-	-	-	-	11.8	260	2	(4)
3′ GMP	11.8	8.0	0.68	0.66	-	-	-	-	11.8	260	2	(4)
5′ GMP (P)	12.1	8.03	0.66	0.	-	-	-	-	13.7	252.5	7	(19, 22
GDP (P)	12.2	7.9	0.65	0.	12.4	8.1	.653	.	13.7	252.5	7	(19, 22
GTP (C)	12.0	8.06	0.67	-	12.3	8.1	.656	.	12.4	257	1	d
d Guo	11.8	7.94	0.67	-	-	-	.	.	13.7	252	7	d
Ura (C)	8.2	1.4	0.175	0.20	-	-	-	-	8.2	260	7	(16)
Urd (C)	9.9	-	0.36	0.35	-	-	-	-	10.0	262	7	(19)
2′ UMP	9.9	3.94	0.398	0.	-	-	-	-	10.0	260	7	(17)
3′ UMP	9.9	3.94	0.398	0.337	-	-	-	-	10.0	260	7	(17)
5′ UMP (P)	9.9	4.05	0.41	0.	-	-	-	-	10.0	262	7	(16)
UDP (P)	9.89	3.82	0.386	0.	9.98	3.78	0.38	0.	10.0	262	7	(16)
UTP (P)	9.87	3.72	0.377	-	9.9	3.77	0.38	0.	10.0	262.	7	(16)
Ino (C)	7.4	1.8	0.24	0.25	-	-	-	-	12.2	248.5	6	(16)
5′ IMP (P)	7.27	1.82	0.25	0.	-	-	-	-	12.2	248.5	6	e
IDP (P)	7.2	1.80	0.25	0.	-	-	-	-	12.2	248.5	6	e
ITP (P)	7.18	1.79	0.25	-	7.21	1.83	0.25		12.2	248.5	6	e

(*table cont'd.*)

For this purpose a solution having a rapid change in absorbance with change in wavelength at 260 mμ, and another having similar characteristics at 280 mμ, are required. The absorbance of these solutions is determined at the band width used in the recording system. In this instance the DB spectrophotometer slit is set at 0.5 mm (manual setting). To obtain a comparable band width for the Beckman DU spectrophotometer a slit setting of 0.81 mm is required. The DU spectrophotometer was calibrated as suggested in the letter circular LC929 from the U. S. National Bureau of Standards.

In practice, four sealed 1-cm silica cuvettes are prepared: two containing 0.1 *N* HCl (a and b), and one each with cytosine (c) and adenine (d) (10 μg/ml) in 0.1 *N* HCl. One blank (b) and the two base-containing cuvettes (c and d) are read against the first blank (a), in the DU spectrophotometer and the results recorded.

To adjust the DB spectrophotometer, (b) is set to read against (a) and the zero adjustment of the DB spectrophotometer moved to match

TABLE 1 (Cont'd.)

pound[a]	pH 4.4				pH 3.6				Reference Data			
	$\alpha_M \times 10^{-3}$ 260	280	Calc.[b] 280/260	Obs.[c] 280/260	$\alpha_M \times 10^{-3}$ 260	280	Calc.[b] 280/260	Obs.[c] 280/260	$\alpha_M \times 10^{-3}$	λ	pH	Reference
(C)	7.40	4.16	0.562	0.55	-	-	-	-	7.8	264	2	(18, 22)
(P)	8.69	6.39	0.735	0.	-	-	-	-	9.65	267	7	(4)
(C)	8.73	6.59	0.755	0.	-	-	-	-	9.7	267	7	(4, 18)
(C)	8.43	6.05	0.718						9.3	267	2	d
Cyt (C)	3.77	7.99	2.12	-	-	-	-	-	9.45	283	2	d
ethd-Cyd (C)	4.47	9.32	2.09	-	-	-	-	-	11.9	286.5	2	d
ethd-CMP (C)	4.06	9.45	2.33	-	-	-	-	-	11.8	287	2	d
oxanthine (N)	8.12	0.85	0.105	-	-	-	-	-	8.1	260	7	(4)

Source of compounds used indicated in parentheses, P = Pabst, C = Calbiochem, N = Nutritional Biochem.

Ratio measured in solutions of commercial preparations.

Ratio observed on peaks off analyzer. Measured on DU at a slit width of 0.81 mm.

Mfg. data.

Inosine constants used.

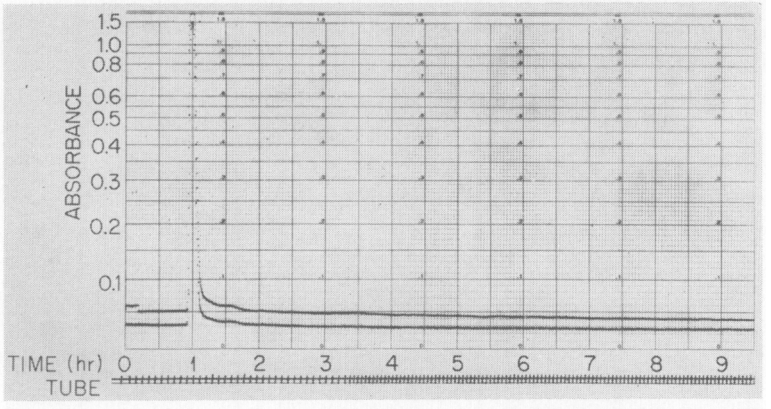

FIG. 11. Elution of bases, nucleosides, and nucleotides from an uncharged resin column, equilibrated and eluted with 0.15 *M* sodium acetate, pH 4.4; 8% cross-linked polystyrene, 200–400 mesh with fines removed, in a 0.9 × 150 cm column; 0.2-cm quartz flow cell. Sample: 0.2 ml each standards A and B, 0.5 ml D, 0.5 ml H, and 1.0 ml I. 99.9% of the added ultraviolet-absorbing material recovered in tubes 11–14. All conditions identical to those used at the start of the long column run described in the text.

the blank readings previously obtained with the DU. With cuvette (c) reading against (a) the 260 setting is adjusted until the indicated absorbance matches that obtained with the DU; similarly, cuvette (d) is used to set the 280 mμ position. The accuracy of the results depends largely on the care with which this calibration is done.

Accuracy of Analysis. The results obtained when the fractions are read manually generally agree within 2% with the results obtained from the automatic recording system. The chief inaccuracy is probably in the flow rate determination, which will be the source of further study.

DISCUSSION

A chromatographic method for resolving mixtures of bases, nucleosides, and nucleotides has been developed for use with an automatic recording system previously described (13). The method exploits the previously recorded observations that nucleotides are not separated on ion-exchange columns solely on the basis of charge, but rather may be constrained by a spectrum of forces that involve not only the phosphate groups but the purine or pyrimidine base and sugar moieties as well (2). These secondary forces, if present, occur only on beads having positively charged groups because no binding to uncharged resin beads has been observed.

By recording the flow rate constantly, great flexibility is introduced into the analytical system. In this work the emphasis has been on high resolution. It is evident that much faster separations can be achieved if higher flow rates and shorter columns are used.

In complex physiological mixtures, considerable overlapping of many of the components must be expected. To separate specific pairs of interest, small changes in pH may be explored. For purely analytical purposes, however, complex peak patterns may often be resolved mathematically into a series of Gaussian curves whose areas and 280/260 ratios may be determined without the necessity of complete physical separation.

SUMMARY

A chromatographic method is described for the separation and determination of nucleotides, nucleosides, and purine and pyrimidine bases using Dowex 1-X8 resin columns 0.9×150 cm in length. Absorbancy at 260 and 280 mμ at optical path lengths of either 0.2 or 1.0 cm were continuously recorded using a modified double-beam spectrophotometer.

To avoid changes in molar absorptivity which occur in many compounds with changing pH, elution was accomplished at one pH (4.4) by changing the concentration of sodium acetate from 0.15 to 3.0 *M*. The complete elution schedule required 28 hr. To shorten the time required

for eluting the triphosphates, a 0.9 × 50 cm column operated at pH 3.6 was used which allowed UTP, ATP, and GTP to be determined in 6 hr.

Note added in proof (C. L. Burger[6] and N. G. Anderson). The chief difficulty in irreproducibility of results owing to resin differences has been solved. After testing batches of Dowex 1-X8 as they were manufactured, the resin characteristics requisite for nucleotide analysis became apparent. Dow Chemical Co., Midland, Michigan, has made a pilot-plant batch of resin which gives essentially the resolution observed in initial studies above. The details of this work and of tissue analyses will be reported elsewhere.

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