CHROM. 4914

THE RAPID SEPARATION OF NUCLEOTIDES IN CELL EXTRACTS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

Nucleotide profiles of cell extracts were determined using high pressure liquid chromatography. The nucleotide levels were quantitatively reproducible and the adenine levels as determined by the analyzer were in close agreement with those determined by an enzymatic cycling procedure. Optimal conditions were determined for the mono-, di- and triphosphates of the naturally occurring ribosides as well as for the sulfur analogs of some of these compounds. The peaks were identified by the use of internal standards, by comparison to chromatograms of standard solutions, by collection and identification of the fractions chemically and spectrophotometrically and by the use of an enzymic peak-shift technique. The latter method which utilizes the specificity of enzyme reactions with a nucleotide or class of nucleotides, can be used not only to verify peak identities but also to clarify or "unmask" a chromatogram. Chromatograms of cell extracts of red blood cells, homogenized schistosomes or murine leukemia or sarcoma cells were obtained in seventy minutes.

INTRODUCTION

In the study of the regulation of metabolic processes and how these processes are affected by drugs, it is important to be able to determine the concentrations of free nucleotides in cell extracts. Until recently, however, there has not been a highly sensitive rapid technique for determining the levels of these various nucleotides.

A liquid chromatographic system developed by Picker Nuclear which combines anion-exchange liquid chromatography under pressure with detection of the peaks by UV spectroscopy has enabled us to determine the nucleotide profile of cell extracts both qualitatively and quantitatively in a short time (70 min). The development of this instrument was made possible by the preliminary work of COHN[1] on ion-exchange chromatography, of ANDERSON[2], who devised a continuous column effluent monitoring system, of KIRKLAND *et al.*[3-5] and of HORVATH *et al.*[6-8], who investigated the operating parameters of high-pressure liquid chromatographic systems.

EXPERIMENTAL

Apparatus

A Picker (now manufactured by Varian) LCS-1000 nucleic acid analyzer was used. The double-beam UV detector operated at 254 mμ. The cylindrical flow cell was 1 mm in diameter and had a 10-mm pathlength. Either a 10-mV or a 1-mV Texas Instrument Servoriter recorder was used. The column, which was purchased from the Varian Corp., is 1.0 mm I.D., 3 m in length and is packed with a pellicular anion-exchange resin. A Gilson Model MF Mini-Escargot fractionator was placed in line to collect the fractions.

Mode of operation

The low-concentration eluent was 0.015 *M* KH_2PO_4 and the high-concentration eluent 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. A mixing chamber in the instrument was filled with low-concentration buffer to the 50-ml mark (the starting volume). The gradient flow rate in which the high concentration buffer was pumped into the mixing chamber was 6 ml/h. The column flow rate or flow of the gradient from the mixing chamber into the column was 12 ml/h. Full scale absorbancy ranges using a 1-mV recorder were from 0.002 to 0.064 absorbancy units and using a 10-mV recorder from 0.02 to 0.64 absorbancy units. For most samples, the UV output of 0.04 or 0.08 was used, although the output can be varied according to the sample size. All samples were introduced with a 10-μl Hamilton syringe.

Chemicals

Reagent grade KH_2PO_4 and KCl were purchased from Mallinkrodt. Standard solutions were prepared from commercially available nucleotides (purchased from P-L Biochemicals, Inc.) or from nucleotides prepared enzymatically in our laboratory. The hexokinase and pyruvate kinase were purchased from Sigma Chemical Co. and the phosphoenolpyruvate from Calbiochem Corporation.

Standard solutions

The standards used as references were the adenine and guanine nucleotides, one solution containing AMP*, ADP and ATP ($\sim$0.5 m*M* in each) and the other GMP, GDP and GTP (also $\sim$0.5 m*M* in each). These solutions were kept at —5° and used qualitatively as references for retention times; however, for quantitation of peaks, freshly prepared solutions were used. Standard solutions of the pyrimidine nucleotides were prepared as well as cyclic 3′5′-AMP and cyclic 2′3′-GMP and 5′-monophosphate nucleotides of xanthine, hypoxanthine, 6-mercaptopurine, 6-methylmercaptopurine and 6-thioguanine.

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* The following abbreviations are used: AMP, ADP, ATP = adenosine 5′-mono-, 5′-di- and 5′-triphosphate; C-AMP = cyclic 3′,5′-adenosine monophosphate; GMP, GDP, GTP = 5′-phosphates of guanosine; C-GMP = cyclic 2′,3′-guanosine monophosphate; UMP, UDP, UTP = 5′-phosphates of uridine; CMP, CDP, CTP = 5′-phosphates of cytidine; TMP, TDP, TTP = 5′-phosphates of deoxythymidine; 6MMPRMP = 5′-monophosphate of 6-methyl mercaptopurineriboside; TGMP = 5′-monophosphate of 6-thioguanosine; XMP = 5′-monophosphate of xanthosine; IMP, IDP = 5′-phosphates of inosine; UDPG = uridine diphosphoglucose; UDPGA = uridine 5′-diphosphoglucuronic acid; NAD, NADH = the oxidized and reduced forms of nicotinamide-adenine dinucleotide.

Identification of peaks

Eluent peaks in cell extracts were identified in the following ways: (1) by collecting the peak and identifying the contents spectrophotometrically or chemically; (2) by injecting known standards along with the sample; (3) by comparing the extract chromatogram to that of known compounds; or (4) by known enzymatic reactions in which an enzyme added to the cell extract specifically converted one of the nucleotides present to another. The latter method showed the simultaneous disappearance of one substrate and the appearance of the corresponding product.

Quantitation of peaks

Since each nucleotide has its own molar extinction coefficient at 254 mμ, separate calibrations were required for quantitating each compound. Areas under the peak were determined by multiplying the height of the peak by the width at half height.

A control experiment was run to determine whether on the column there is any retention of compounds with subsequent bleeding out of these substances in later runs (ghosting). For this experiment a standard solution of the adenine nucleotides was run. The fractions and the effluent from the low concentrate wash were collected at 2½-min intervals. A cell extract containing radioactive [^{14}C]adenine and [^{14}C]guanine nucleotides was then injected into the column and the effluent containing this sample was collected. The subsequent wash was collected separately. Another standard solution of adenine nucleotides was then run and this standard, and the wash after it, collected. All the collection tubes were washed with Bray's solution and the solution transferred to counting vials. The wash solutions and the standard solution run following the ^{14}C sample were found to have only background levels of radioactivity. A plot of the counts of the radioactive sample corresponded to the chromatogram from the analyzer (Figs. 1 and 2). Therefore, it was concluded that there is no observable retention of the nucleotides on the column and that distribution of the peaks determined by radioactivity corresponds well to the distribution of peaks as determined by UV absorption.

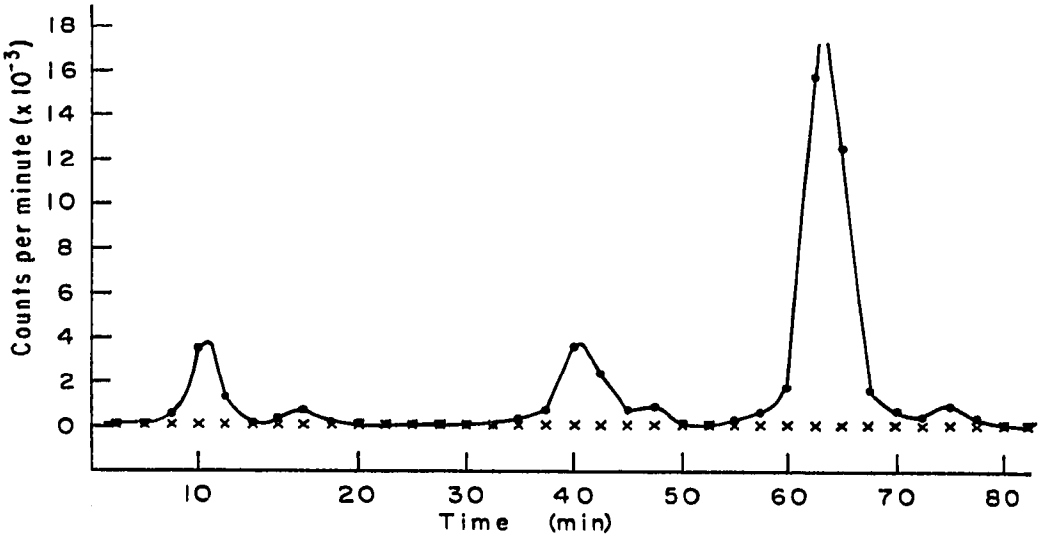

Fig. 1. Plot of counts (^{14}C) per minute *vs.* time of the schistosome sample. The sample is designated by dots and the control and washes by crosses.

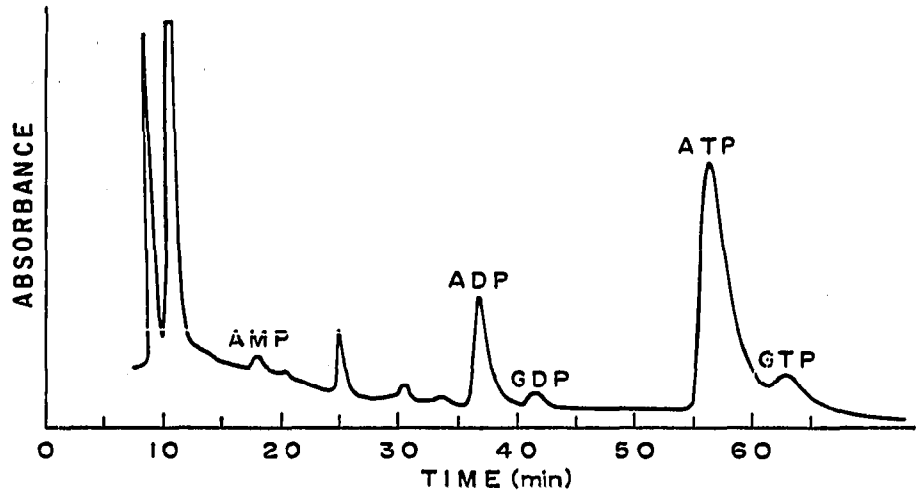

Fig. 2. Separation of nucleotides from homogenate of schistosomes. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 20 μl of extract of homogenate of schistosomes; UV output: 0.04.

RESULTS AND DISCUSSION

To obtain the optimum separation of nucleotides, a few operating parameters can be predicted, but most must be obtained by trial and error. The types of eluents used in conventional ion-exchange chromatography may also be used in a pellicular resin column under pressure, but with the latter methods the eluents are much less concentrated. The effect of temperature, flow rate, mode of elution, pH and salt concentration of the eluents has been discussed in detail for the separation of nucleosides and bases on a cation-exchange column by HORVATH AND LIPSKY[8], and for nucleotides on an anion-exchange column by HORVATH *et al.*[7]. In the present study, it was found that temperature and variations in pH (from 3.5–5.6) are less important than are the flow rates and salt concentration for optimizing the sharpness and separation of peaks.

Standards

Standard adenine and guanine nucleotides were analyzed routinely several times a week to check the reproducibility of the results and the stability of the instrument and column conditions. A representative chromatogram of these nucleotides is shown in Fig. 3. Fig. 4 shows a chromatogram of some of the important naturally occurring purine and pyrimidine nucleotides. The retention times of these standard solutions are given in Table I. It should be noted that retention times may vary from instrument to instrument and from column to column. Minor variations in retention times may also be caused by column conditions, fluctuations in the hydraulic system, and slight changes in purging procedure between runs. However, the relative order of peaks did not change and the small variations in retention times were correlated to the standard adenine and guanine nucleotide solutions which were run routinely. The order of the nucleotides is characteristically C, U, T, A and G for the mono- and diphosphates as well as the triphosphate nucleotides. Cyclic 3',5'-AMP and cyclic 2',3'-GMP had

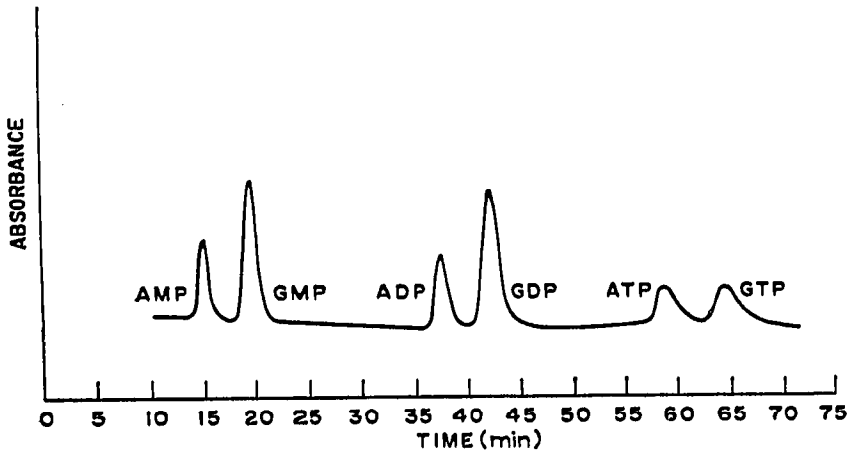

Fig. 3. Separation of mono-, di- and triphosphates of adenosine and guanosine. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; samples: 3 μl of a mixture of ~ 0.05 m*M* AMP, ADP and ATP and 3 μl of a mixture of ~ 0.05 m*M* GMP, ADP and GTP; UV output: 0.08.

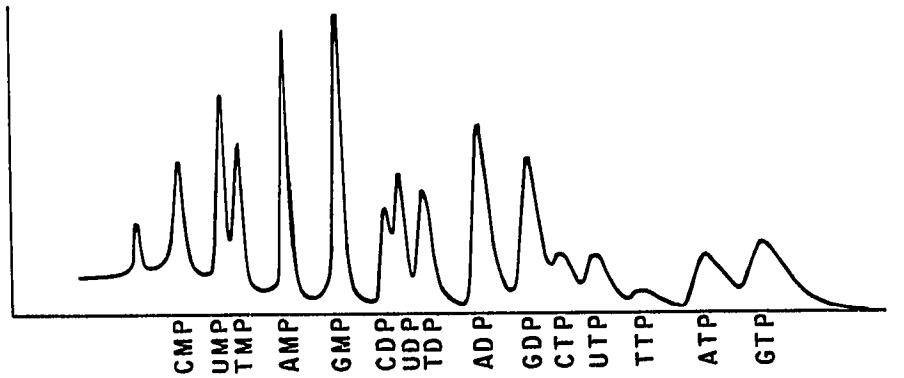

Fig. 4. Separation of mono-, di- and triphosphates of purine and pyridine ribosides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 2 μl of a solution of adenine nucleotides (~ 0.5 m*M* in each), 2 μl of a solution of guanine nucleotides (~ 0.5 m*M* in each), 2 μl of a solution of cytidine nucleotides (~ 0.2 m*M* in each), 2 μl of a solution of uridine nucleotides (~ 0.2 m*M* in each), and 2 μl of a solution of thymidine nucleotides (~ 0.2 m*M* in each); UV output: 0.08.

TABLE I

RETENTION TIMES (min) OF STANDARD NUCLEOTIDE SOLUTIONS

Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; UV output: 0.08.

Monophosphates		*Diphosphates*		*Triphosphates*	
CMP	10	NAD	8½	CTP	46
UMP	12	UDPG	24	UTP	49½
TMP	14	CDP	29	TTP	54
IMP	18	UDP	31	ATP	60
AMP	19	TDP	33	GTP	65
GMP	23	ADP	38		
C-AMP	24	NADH	39		
6MMPRMP	27	GDP	43		
TGMP	32	UDPGA	44		
XMP	34				

longer retention times than their linear analogs (Figs. 5a and b). This order of retention has also been found in conventional column chromatography using an anion-exchange column[9].

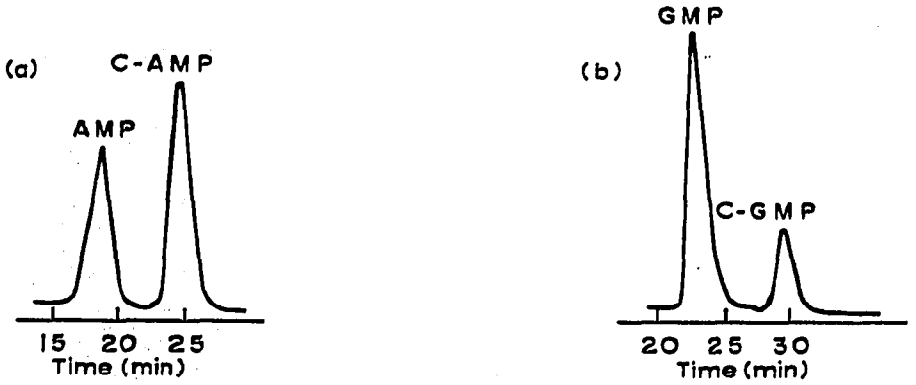

Fig. 5. Separation of cyclic AMP and GMP from their linear analogs. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; samples: (a) 4 μl AMP and 1 μl C-AMP, (b) 4 μl GMP and 1 μl C-GMP; UV output: 0.16.

Optimal operating conditions

In order to find the optimal operating conditions for cell extracts, chromatograms of the adenine and guanine nucleotides were obtained in which the following conditions were varied one at a time: (1) temperature, (2) flow rates, (3) starting volume of low-concentrate eluent, (4) pH of eluent, (5) eluent composition, and (6) sample size (both volume and concentration).

Temperature, flow rates, starting volume, pH and salt concentration

It was found that 75° was the optimal temperature for peak sharpness, especially with the triphosphate nucleotides. Higher temperatures were not tried because of the well-known heat lability of di- and triphosphate nucleotides. The best resolution was possible using a column flow rate of 12 ml/h and a gradient flow rate of 6 ml/h. Increasing both the gradient and column flow rates increased peak sharpness and reduced retention time. These advantages, however, were offset by a disadvantage: these chromatograms were so crowded that there was not enough space in the cell extract chromatograms for good separation of all purine and pyrimidine nucleotide peaks (Fig. 6). The optimal starting volume of low-concentration buffer was 50 ml. Lowering the starting volume had the same advantages and disadvantages as increasing the flow rates. Changing the pH of the eluents affected the separation and peak sharpness, but to a much lesser degree. The optimal pH of both eluents for our work was 4.5. The salt concentration had a significant effect in both peak sharpness and resolution. It was found that the solutions suggested by SCHMUCKLER[10] of 0.015 *M* KH_2PO_4 in 2.2 *M* KCl were best for our purposes. Although formate solutions are used routinely in anion-exchange column chromatography, it has not been used in this instrument because of possible reaction of the formate with the stainless steel components[8].

Sample size

Sample volumes of 2 to 20 μl were injected into the column. Since we are limited to using a 10-μl syringe because of the instrument design, any volume larger than

10 μl had to be injected twice, thus doubling the possibility of sampling errors. Sample solution volumes in the range of 2 to 9 μl were the most satisfactory, giving the best quantitatively reproducible results. Solutions of nucleotides ranging from 0.01 mM to 1.0 mM were used with the natural nucleotides, and it was found that the concentrations were proportional to peak area. A plot of concentration *vs.* area is shown in Fig. 7.

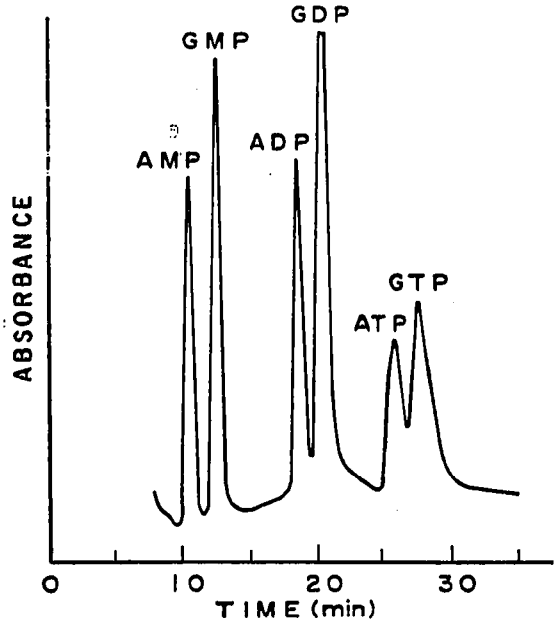

Fig. 6. Separation of adenine and guanine nucleotides with low starting volume of low-concentrate eluent and high flow rates. Starting volume: 35 ml; flow rates; 24 ml/h (gradient into column) and 12 ml/h (high-concentrate eluent into low); eluents: 0.015 M KH_2PO_4 and 0.25 M KH_2PO_4 in 2.2 M KCl; samples: 1 μl of a solution of adenine nucleotides and 1 μl of a solution of guanine nucleotides; UV output: 0.08.

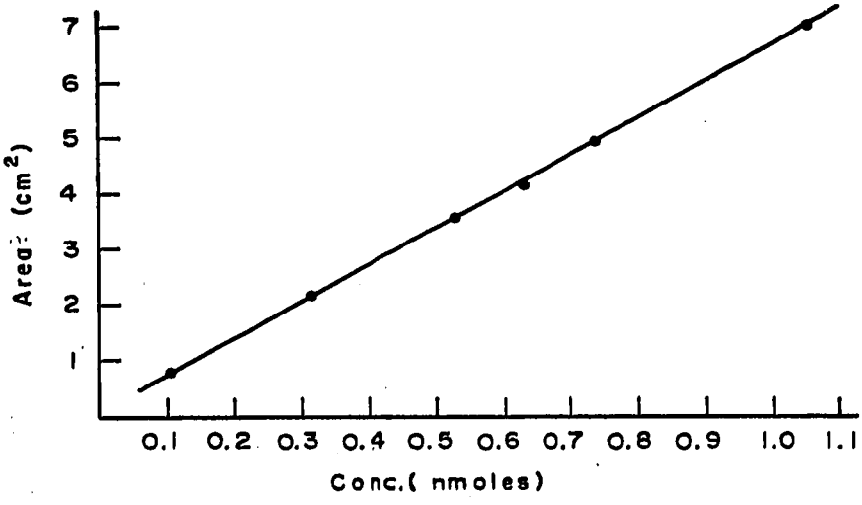

Fig. 7. Plot of concentration (in nmoles) of AMP *vs.* area (in cm^2) of AMP peak.

Eluents for constant eluent strength mode

For complete cell extracts, the gradient elution mode was used, but for the determination of either the mono-, the di- or the triphosphate nucleotides alone, the samples were run using a single concentration of eluent. For the separation of AMP and GMP, a solution of 0.10 *M* KCl gave good resolution; for ADP and GDP, 0.015 *M* KH_2PO_4 in 0.15 *M* KCl; and for ATP and GTP, 0.019 *M* KH_2PO_4 in 0.19 *M* KCl. The retention times were as follows: AMP, 11 min; GMP, 14½ min; ADP, 10 min; FDP, 12 min; ATP, 12 min; GTP, 16½ min.

Quantitation

In the quantitation of the adenine and guanine nucleotides, ten samples each of a standard solution of AMP and of GMP were run. The areas which were calculated by multiplying the height times the width at half height are tabulated in Table II.

TABLE II

TABULATION OF AREAS UNDER THE PEAKS OF STANDARD SOLUTIONS OF AMP AND GMP

The AMP solution was 6.08×10^{-5} *M* and the GMP solution was 5.85×10^{-5} *M*. The volume of sample solution injected ranged from 6–8 µl. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; UV output: 0.08.

AMP		*GMP*	
Spectrum No.	*Area/nmole*	*Spectrum No.*	*Area/nmole*
163	6.42	147	6.48
171	6.81	148	6.24
173	6.74	149	6.64
174	6.87	150	6.66
175	6.92	152	6.41
176	6.49	153	6.39
180	6.76	154	6.57
181	6.81	155	6.54
182	6.52	156	6.60
183	6.69	158	6.63
Mean	6.71	Mean	6.52
Standard deviation	0.18	Standard deviation	0.10
Coefficient of variation	2.8	Coefficient of variation	1.5

The standard deviation for AMP was 0.19 and for GMP was 0.10; the coefficient of variation for AMP was 2.6 % and for GMP, 1.5 %. The concentration of all adenine nucleotides in cell extracts was calculated by relating peak areas to that of the standard AMP and of all guanine nucleotides to that of GMP. A comparison of values of total adenine nucleotide content as determined by the analyzer and by an enzymatic method in four different blood samples showed close agreement and is shown in Table III. Samples were assayed by Dr. RALPH MIECH by an enzymatic cycling procedure specific for adenine nucleotides which employs ATP:GMP phosphotransferase, ATP:AMP phosphotransferase, pyruvate kinase and lactic acid dehydrogenase. Details will be published elsewhere.

TABLE III

TOTAL ADENINE NUCLEOTIDES (AMP + ADP + ATP) (nmoles/μl)

	Sample			
	H	*P*	*A*	*S*
Analyzer	0.387	0.344	0.354	0.229
Enzymatic analysis	0.393	0.332	0.348	0.230

Cell extracts

The free nucleotide content of cell extracts of murine Sarcoma 180 cells, murine leukemia cells (L5178Y), homogenized *Schistosoma mansoni* and human and rat red blood cells were determined. As in all nucleotide analyses, care in the preparation of the extract is of utmost importance in order to minimize the degradation of the nucleotides and to obtain valid results[11]. Samples of the chromatograms obtained in the study of the effect of purine antimetabolites on nucleotide levels of Sarcoma 180 cells are shown in Figs. 8 and 9. Fig. 2, as was noted previously in the control experiment for retention of nucleotides on the column, is typical of the chromatograms of extracts of homogenized schistosomes. Fig. 10 shows a chromatogram obtained in the study of the effect of antimetabolites on murine leukemia cells; Fig. 11 is representative of the identification of peaks in a chromatogram by the addition of known standard solutions. In this case, a solution of the uridine nucleotides was the standard used.

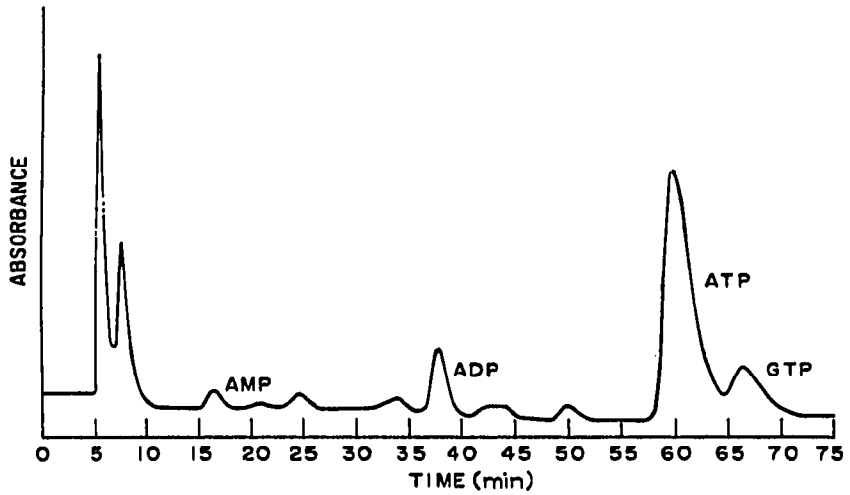

Fig. 8. Separation of nucleotides in cell extract of Sarcoma 180 cells. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 8 μl of a perchloric acid extract of Sarcoma 180 cells; UV output: 0.04.

"Enzymic peak-shifts"

"Enzymic peak-shifts" were used as a method of verifying peak identities. This technique utilizes the specificity of enzyme reactions with a nucleotide or class of nucleotides. An example of this procedure is the use of hexokinase (HK) and an excess of glucose to identify the ATP and ADP peaks[12].

$$\text{ATP} + \text{glucose} \xrightarrow[Mg^{2+}]{HK} \text{ADP} + \text{glucose-6-phosphate}$$

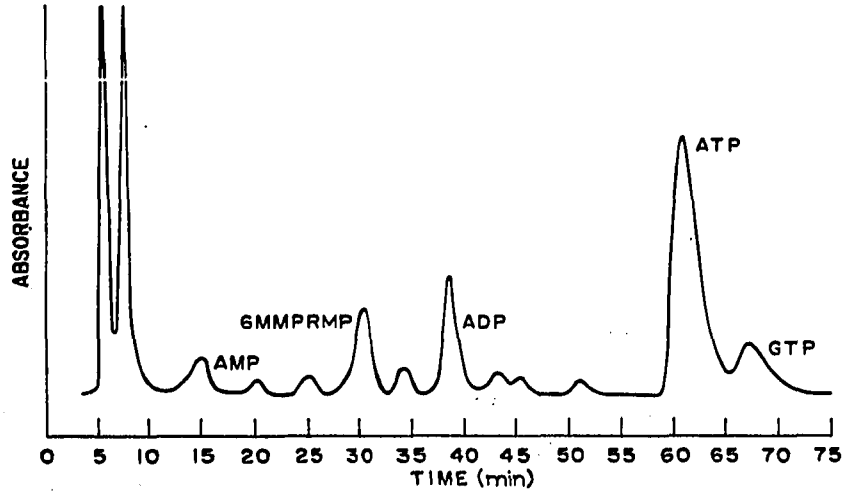

Fig. 9. Separation of nucleotides in extracts of Sarcoma 180 cells which had been treated for 1 h with an antimetabolite. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 8 μl of perchloric acid extract of drug-treated Sarcoma 180 cells; UV output: 0.04.

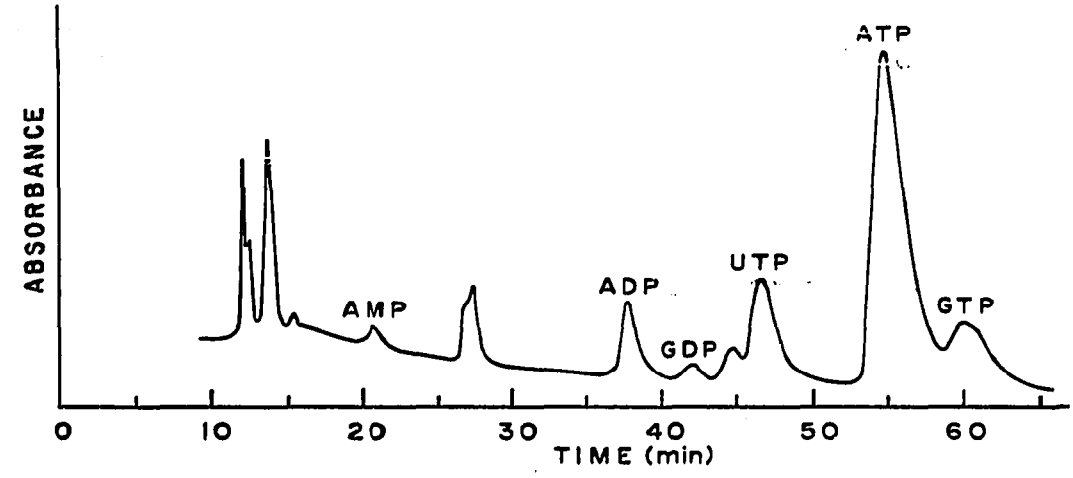

Fig. 10. Separation of nucleotides in a TCA extract of tissue culture of murine leukemia cells (L5178Y). Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 4 μl; UV output: 0.04.

In this reaction, the ATP peak disappeared and the ADP increased proportionally in size. The peaks for the guanine nucleotides and the AMP were not altered. Therefore, positive identification is established for the ATP and ADP peaks. In all the enzymic peak-shift reactions, the solutions were treated with trichloroacetic acid (TCA) to precipitate any protein or acid-insoluble material and to prevent accumulation of enzyme on the column. The nucleotides were in the TCA supernatant. The TCA was then removed by extraction with water-saturated diethyl ether. The chromatograms of standard solutions of extracted adenine and guanine nucleotides before and after the reaction with hexokinase are shown in Figs. 12 and 13. The same nucleotides were

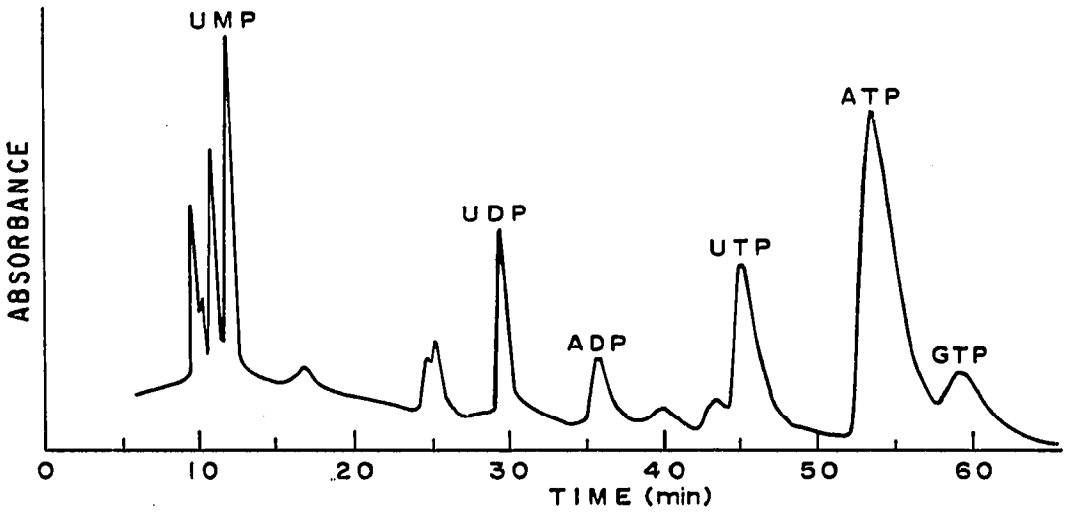

Fig. 11. Identification of uridine peaks by the addition of uridine nucleotides to a TCA extract of a tissue culture of murine leukemia cells (L5178Y). Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; samples: 4 μl cell extract and 1 μl solution of uridine nucleotides (mono-, di-, and triphosphate); UV output: 0.04.

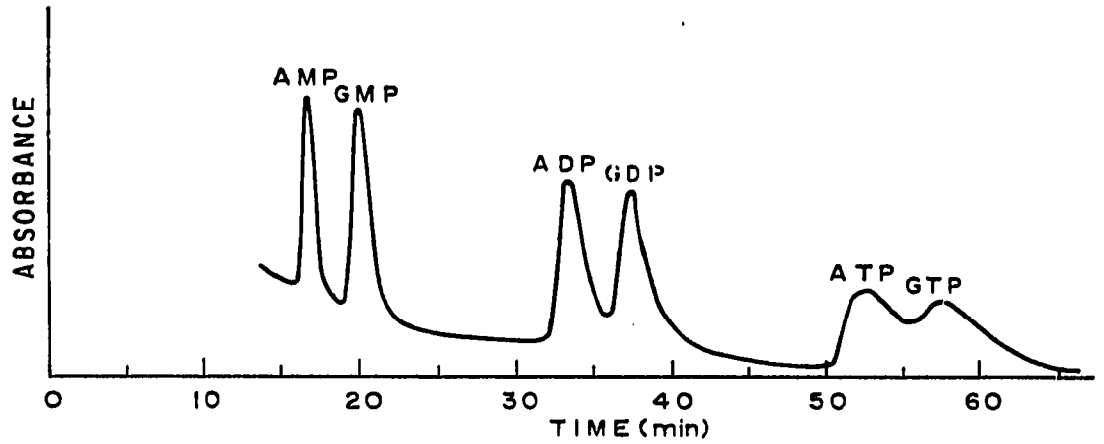

Fig. 12. Separation of a TCA extract of adenine and guanine nucleotides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents; 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 10 μl of a TCA extract of solutions of AMP, ADP and ATP and of GMP, GDP and GTP; UV output: 0.32 (1-mV recorder).

treated with pyruvate kinase (PK) and an excess of phosphoenolpyruvate (PEP) in the presence of magnesium chloride[13].

$$ADP + PEP \xrightarrow[Mg^{2+}]{PK} ATP + \text{pyruvate}$$

$$GDP + PEP \xrightarrow[Mg^{2+}]{PK} GTP + \text{pyruvate}$$

In this reaction, the diphosphates are phosphorylated to 5'-triphosphate nucleotides Fig. 14 is the chromatogram of an extract of a solution mixture after adenine and guanine nucleotides had been incubated with PK and PEP for 10 min. There are no

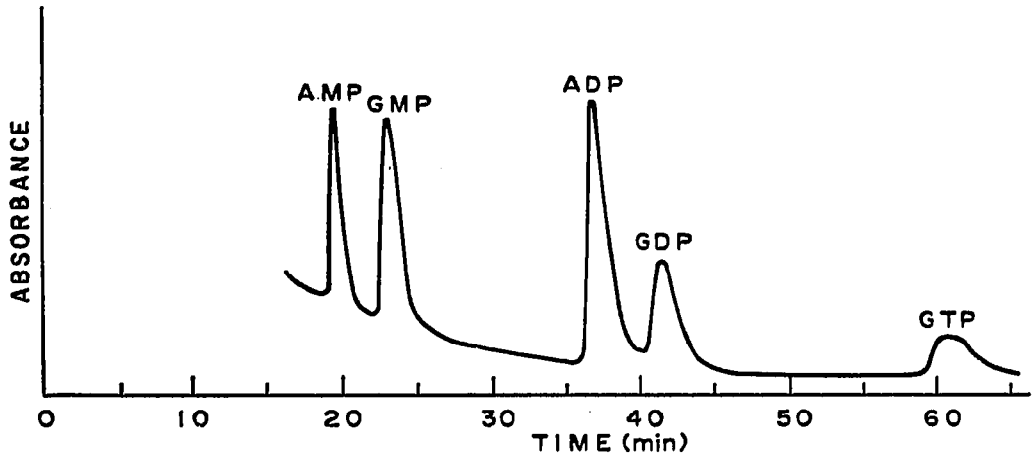

Fig. 13. Peak shifts caused by reaction of hexokinase and glucose on a solution of adenine and guanine nucleotides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 10μl of a TCA extract of reaction mixture; UV output: 0.32 (1-mV recorder).

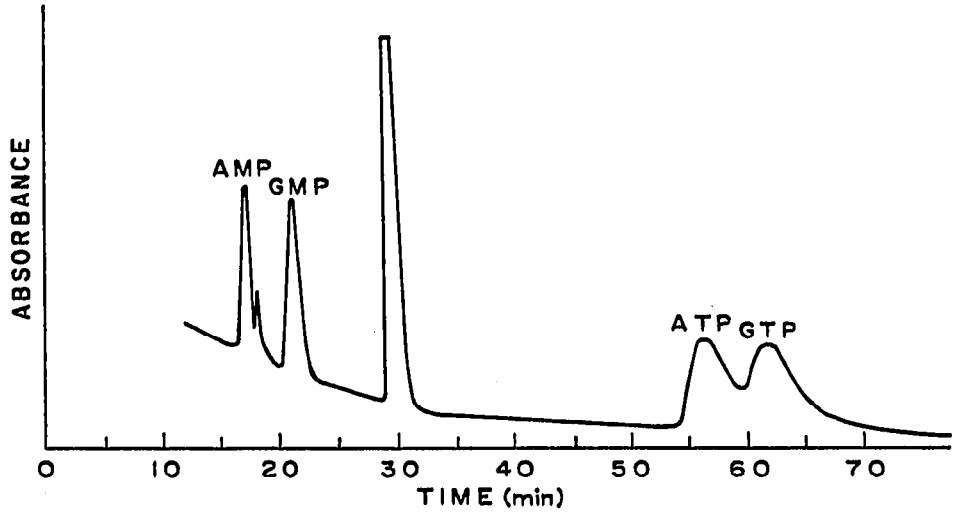

Fig. 14. Peak shifts caused by reaction of pyruvate kinase and phosphoenolpyruvate on a solution of adenine and guanine nucleotides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; Sample: 10 μl of a TCA extract of reaction mixture; UV output: 0.32 (1 mV).

diphosphate nucleotide peaks and the triphosphate nucleotide peaks have increased in size. The peak which has a retention time of 30 min may be PEP, but it has not been positively identified as yet.

"Enzymic peak-shift" reactions such as these are also useful in clarifying or "unmasking" a chromatogram. When one nucleotide is present in large quantity, it may hide the presence of small quantities of other nucleotides which have similar retention times. By completely removing the large peak, we are then able to show conclusively the nucleotides that are present. This technique is also helpful in the quantitation of a hidden peak or in determining the shape of a peak which otherwise can only be seen as a shoulder.

TABLE IV

ENZYMES THAT CAN BE USED IN "ENZYMIC PEAK-SHIFT" METHOD OF IDENTIFYING PEAKS IN CELL EXTRACT CHROMATOGRAMS

Trivial names	*EC No.*	*Systematic names*
Adenylate kinase	(2.7.4.3)	ATP:AMP phosphotransferase
ADP deaminase	(3.5.4.7)	ADP aminohydrolase
AMP deaminase	(3.5.4.6)	AMP aminohydrolase
Apyrase	(3.6.1.5)	ATP diphosphohydrolase
ATPase	(3.6.1.5)	ATP phosphohydrolase
Creatine kinase	(2.7.3.2)	ATP:creatine phosphotransferase
Deoxy-CMP deaminase	(3.5.4)	Deoxy-CMP aminohydrolase
Hexokinase	(2.7.1.1)	ATP:D-hexose 6-phosphotransferase
IMP cyclohydrolase	(3.5.4.10)	IMP 1,2-hydrolase
Pyruvate kinase	(2.7.1.40)	ATP:pyruvate phosphotransferase
UDPG dehydrogenase	(1.1.1.22)	UDPG glucose: NAD oxidoreductase

Other possible enzyme reactions

It is possible to use many other enzyme reactions in the "enzymic peak-shift" method of identifying nucleotide peaks. Almost any of the phosphotransferases (in the groups numbered 2.7.1 to 2.7.4 by the Enzyme Commission)* can be used with an excess of the appropriate substrate resulting in the disappearance of the ATP peak and the increase of the ADP peak[14]. The pyrophosphotransferases (2.7.6) can identify ATP and AMP and certain nucleotidylotransferases (2.7.7) react specifically with ATP, ADP, CTP, GDP, GTP, dTTP or the various sugar analogs of the uridine triphosphate nucleotides[14]. Some of the enzymes which act on acid anhydride bonds (3.6.1) such as ATPase and apyrase are also applicable for use in location on the chromatogram of ATP, ADP and AMP. The same is true for deoxy-CTPase for dCTP and dCMP[14].

AMP deaminase, ADP deaminase and deoxy-CMP deaminase (of the classification 3.5.4) can serve to characterize AMP and IMP, ADP and IDP, and dCMP and dUMP[14]. Uridine 5'-diphosphoglucose dehydrogenase with UDPG and NAD can be used to characterize not only the reactants, UDPG and NAD^+, but also the products, UDPGA and NADH[15].

In the case of a reversible reaction, this reaction can be coupled to a second enzymatic reaction to remove a product of the first reaction. If the second reaction is exothermic, the first reaction is driven to completion. If the second reaction is readily reversible, its equilibrium can be shifted to the right by adding an excess of substrate. A requirement in all such reactions is that neither the reactants nor the products absorb in the UV at 254 mμ.

One example of coupled enzymatic reactions is the reaction of ADP in the presence of adenylate kinase (AK) to form AMP and ATP[16], which can be driven to

* See Table IV for trivial and systematic names, and Enzyme Commission numbers of enzymes used in "enzymic peak-shifts".

completion in the presence of AMP deaminase. Since AMP deaminase converts AMP to IMP and NH_3[17], the products of the combined reactions are ATP, IMP, and NH_3.

$$2\ ADP \overset{AK}{\rightleftarrows} AMP + ATP$$

$$AMP \xrightarrow[\text{deaminase}]{AMP} IMP + NH_3$$

$$2\ ADP \xrightarrow[AMP]{AK} ATP + IMP + NH_3$$
deaminase

Another procedure which involves the reaction of a product of the reversible reaction with another enzyme and excess substrate is the use of hexokinase (HK) and an excess of glucose in conjunction with the adenylate kinase reaction[12]. The ATP produced reacts with glucose until no ATP remains; thus, AMP and glucose-6-phosphate (G-6-P) accumulate.

$$2\ ADP \overset{AK}{\rightleftarrows} ATP + AMP$$

$$ATP + glucose \xrightarrow{HK} ADP + G\text{-}6\text{-}P$$

$$ADP + glucose \xrightarrow[HK]{AK} AMP + G\text{-}6\text{-}P$$

In both cases, these reactions are specific for the disappearance of the ADP. In the former reaction, two nucleotides accumulate, ATP and IMP, while in the latter only AMP is produced.

A third example of driving a reversible reaction in the desired direction by coupling it to another enzymatic reaction is the reverse of the previous reaction, the conversion of AMP and ATP in the presence of adenylate kinase to ADP. In the presence of creatine kinase (CK) and an excess of phosphocreatine[18], the reactions continue until there is no AMP or ADP in the reaction mixture; thus only ATP, phosphocreatine and creatine remain.

$$AMP + ATP \overset{AK}{\rightleftarrows} 2\ ADP$$

$$2\ ADP + 2\ phosphocreatine \overset{CK}{\rightleftarrows} 2\ ATP + 2\ creatine$$

$$AMP + 2\ phosphocreatine \xrightarrow[CK]{AK} ATP + 2\ creatine$$

Other coupled reactions for enzymic peak-shifts are possible, but the reactions outlined are especially applicable because of the high specificity of these enzymes for the adenine nucleotides. The reagents are readily available and inexpensive and none

of the added substrates or conversion products absorb in the UV at 254 mμ. In most cellular extracts, the concentrations of the adenine nucleotides are much greater than those of other nucleotides. Thus, "enzymic peak-shift" methods for the adenine nucleotides may be of especial value in "unmasking" peaks of the non-adenine compounds ordinarily hidden by the larger adenine nucleotide peaks. Work is proceeding to obtain the best possible conditions for carrying out these reactions and other conversions which can be used to characterize as many as possible of the nucleotide peaks.

Use of isotopes

The high-pressure liquid chromatographic system can be used in combination with isotopes for measuring radiospecific activities of nucleotides. This was demonstrated in the work with schistosomes in which the schistosomes were incubated with ^{14}C-labeled precursors to determine whether salvage pathways or *de novo* pathways were employed by these parasites in the metabolism of nucleotides[19]. In the experimental treatment of murine leukemia cells with antimetabolites, ^{35}S was incorporated into the drugs in order to determine both by the specific activity and the nucleotide profiles whether these drugs interfered with the metabolic processes at the nucleotide or the nucleic acid level. In the study of the effect of cancer chemotherapeutic agents on the Sarcoma 180 cells, time studies of the formation of the metabolites were carried out by means of paper chromatography and isotopes while the adenine and guanine nucleotide levels were determined quantitatively by the high-pressure liquid chromatographic system.

Limitations and possible improvements

A major limitation of this system is the UV detector which is only available at one set wavelength (254 mμ). In order to study analog nucleotides, especially 6-thio and 6-seleno purines (which absorb from 290–370 mμ), a second wavelength would be very useful. Other possible improvements include incorporation into the analyzer of a flow scintillation counter so that isotope studies could be carried out simultaneously with the separation. The development of procedures that would allow cell extracts to be run on a straight elution mode rather than a gradient would save the time now needed to condition the column between runs. It is not yet possible to distinguish between the deoxyribonucleotides and the ribonucleotides under the conditions we have used. A commonly employed device for solving this problem involves the use of borate ion in the elution system which would complex with the ribonucleotides and modify their migration[20,21]. Although we have not tested this method, it seems likely that separations can be effected using this technique.

In conclusion, high-pressure liquid chromatography can be an invaluable tool in the study of cell extracts. For non-volatile, polar compounds, it approaches the speed, efficiency and sensitivity that gas chromatography has for volatile, non-polar substances. This technique shortens the time and tedium previously involved in determining nucleotide levels. Therefore, it is possible to obtain quickly and easily nucleotide profiles which are qualitatively and quantitatively reproducible.

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REFERENCES

1 W. E. COHN, *J. Am. Chem. Soc.*, 72 (1950) 1471.
2 N. G. ANDERSON, *Anal. Biochem.*, 4 (1962) 269.
3 J. J. KIRKLAND, *Anal. Chem.*, 40 (1968) 391.
4 J. J. KIRKLAND, *Anal. Chem.*, 41 (1969) 218.
5 J. J. KIRKLAND AND H. FELTON, *J. Chromatog. Sci.*, 7 (1969) 7.
6 C. HORVATH AND S. LIPSKY, *Nature*, 211 (1966) 748.
7 C. HORVATH, B. PREISS AND S. LIPSKY, *Anal. Chem.*, 39 (1967) 1422.
8 C. HORVATH AND S. LIPSKY, *Anal. Chem.*, 41 (1969) 1227.
9 G. NORTHROP, *Ph. D. Thesis*, University of Wisconsin, 1963, p. 23.
10 H. SCHMUCKLER, personal communication.
11 P. MANDEL, in J. DAVIDSON AND N. COLEN (Editors), *Progress in Nucleic Acid Research and Molecular Biology*, Vol. III, Academic Press, New York, 1964, pp. 304–306.
12 H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 543.
13 H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 573.
14 M. DIXON AND E. WEBB, *Enzymes*, 2nd Ed., Academic Press, New York, 1964, pp. 716–725.
15 E. MAXWELL, H. KALCKAI AND J. STROMINGER, *Arch. Biochem. Biophys.*, 65 (1956) 2.
16 L. NODA AND S. KUBY, *Methods Enzymol.*, 6 (1963) 223.
17 A. MUNCH-PETERGEN AND H. KALCKAI, *Methods Enzymol.*, 6 (1963) 869.
18 S. KUBY, L. NODA AND H. LARDY, *J. Biol. Chem.*, 202 (1953) 217.
19 A. W. SENFT, R. P. MIECK, P. R. BROWN AND D. SENFT, *De Novo or Solvage Pathway in Purine Metabolism in Schistosoma mansoni*, in press.
20 J. X. KHYM AND W. E. COHN, *Biochim. Biophys. Acta*, 15 (1954) 139.
21 W. E. COHN AND F. J. BOLLUM, *Biochim. Biophys. Acta*, 48 (1961) 588.