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# The quantitative separation of 3',5'-cyclic adenosine monophosphate, adenosine-5'-monophosphate, adenosine-5'-diphosphate, and adenosine-5'-triphosphate by ion-exchange chromatography on diethylaminoethyl Sephadex

During the course of an investigation into the degradation of adenine nucleotides by mammalian skin we needed a method for the quantitative separation of the various adenine nucleotides from each other that would involve a minimum of time and manipulation with a maximum of ease. The following paper describes the procedures that we have developed which partially fulfill some of these requirements.

Separations of adenine nucleotides by means of column chromatography on various ion exchangers have been previously described by BRADHAM, ÖCKERMAN, COHN[1-3] and many others. The theory of systematic variation of elution gradients has been described by BOCK and others[4,5]. Our latest methods involve an integration and modification of the above procedures. In brief, we are able to separate quantitatively, with good recoveries, 3'5'-cyclic adenosine monophosphate (CAMP), adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP), and adenosine-5'-triphosphate (ATP), by use of a concave gradient of ammonium bicarbonate (0–0.4 $M$), on a column of diethylaminoethyl Sephadex (DEAE-Sephadex).

*Experimental*

*Materials.* DEAE-Sephadex A-25 (medium) was obtained from the Pharmacia Company and prepared as recommended by them in the chloride form with the following exception: Fines were removed by repeated decantation of the gel after swelling but prior to the alkaline and acid wash.

The number of decantations varied but was generally greater than ten. This was done in order to prevent excessive back pressure on the column while the eluants were being pumped through at high rates of speed. The various nucleotides were obtained from Sigma Company. All solutions were made up in deionized glass distilled water.

*Apparatus.* The chromatography columns used were 10.5 × 250 mm glass columns fitted with a coarse sintered glass disk at the bottom. The top of the column was plugged with a No. 00 rubber stopper pierced by No. 19 stainless-steel hypodermic needle. All connections were made with intramedic polyethylene tubing (PE-100 obtained from Clay Adams Co.) attached to standard luer or luer lock connectors, with the exception of the connection between the gradient vessels, which was 0.25-in. rubber tubing. The gradient vessels were prepared by boring ¼-in. holes at the sides of the bases of nalgene graduated cylinders. The larger vessel was a 1-l cylinder with an I.D. of 62 mm. The smaller cylinder had a capacity of 500 ml and an I.D. of 46 mm. Disposable plastic polyethylene syringes (1.0 ml) were cut at the 0.6-ml mark and forced into the ¼-in. holes bored in the cylinders. The edges were sealed by use of a Sears-Roebuck hot melt glue gun. This gave inexpensive luer connectors at the base of the gradient vessels (Fig. 1). The pump used was a Milton Roy Model 196-47 metering pump, which has a maximum pumping capacity of 240 ml/h. Various fraction collectors were used but it was found that those fitted with drop counting were the
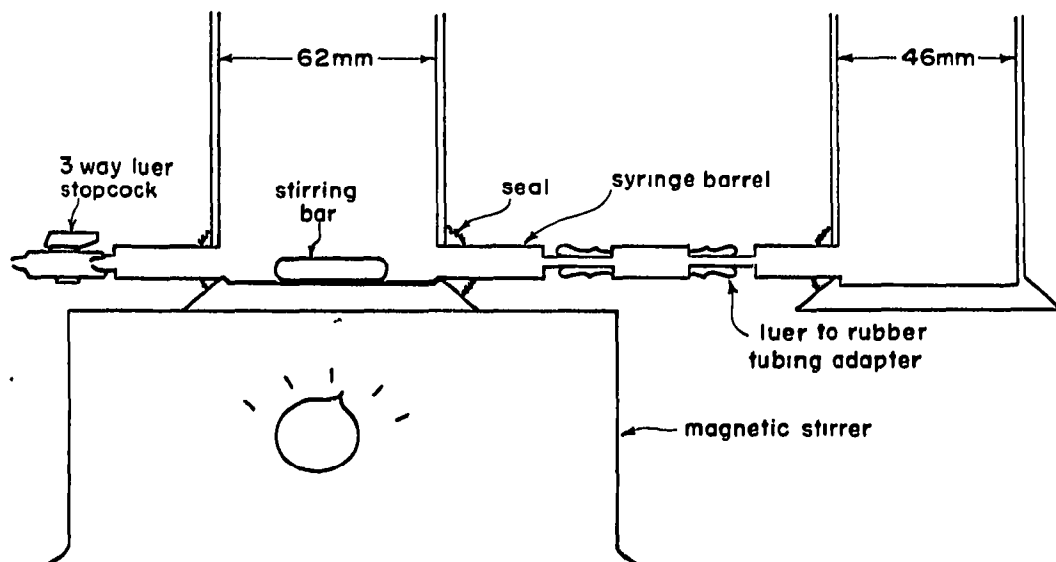
Fig. 1. A schematic representation of the gradient apparatus

most efficient since changes in the ionic strength of the elution medium caused shrinking of the gel and this in turn varied the back pressure on the pump.

*Separation procedures.* Separation of the four nucleotides was accomplished as follows: Mixtures of known amounts of known nucleotides were titrated to pH 7.0 and then diluted to 5 ml. This volume was then pumped through the column four successive times, the original container being washed into the column each time. The material emerging from the column after the four successive passes essentially had no optical density at 257 m$\mu$. The column was then washed with 100 ml of distilled water and the gradient commenced by removing the clamp from the rubber tubing connecting the two cylinders. The gradient consisted of 660 ml of distilled water in the wide cylinder and 340 ml of 0.4 $M$ ammonium bicarbonate in the smaller cylinder. This gives an approximately 0.25-in. difference in heights of liquid, which is sufficient to offset the greater density of the ammonium bicarbonate solutions. A magnetic stirrer was placed in the larger cylinder and adjusted to constant speed. The fraction collector was set to collect 100 10-ml fractions. The pump was set at its maximum speed of 240 ml/h. The total separation procedure generally takes 4.5 h Peaks are detected by reading optical density at 257 m$\mu$ of aliquots from each tube (generally 0.5 ml added to 2.5 ml of water in a 1.0-cm 3-ml quartz cuvette).

*Determination of recoveries.* Where recoveries were being determined, the same amounts of known nucleotides were added first to the titration vessel and then to a volumetric flask (250 ml) using the same pipette in the same manner. To each of the volumetric flasks 25 ml of 0.4 $M$ ammonium bicarbonate were added and then they were made up to volume. After mixing, optical density was determined on a portion of each of the volumetric flasks and then the amounts added were calculated. After collection of fractions and determinations of peaks, each peak was carefully washed into a flask, made up to volume and the optical density of an aliquot read. The optical densities of the portions of each peak removed from the fraction collector tubes to determine the position of these peaks were added up and their sum added to the
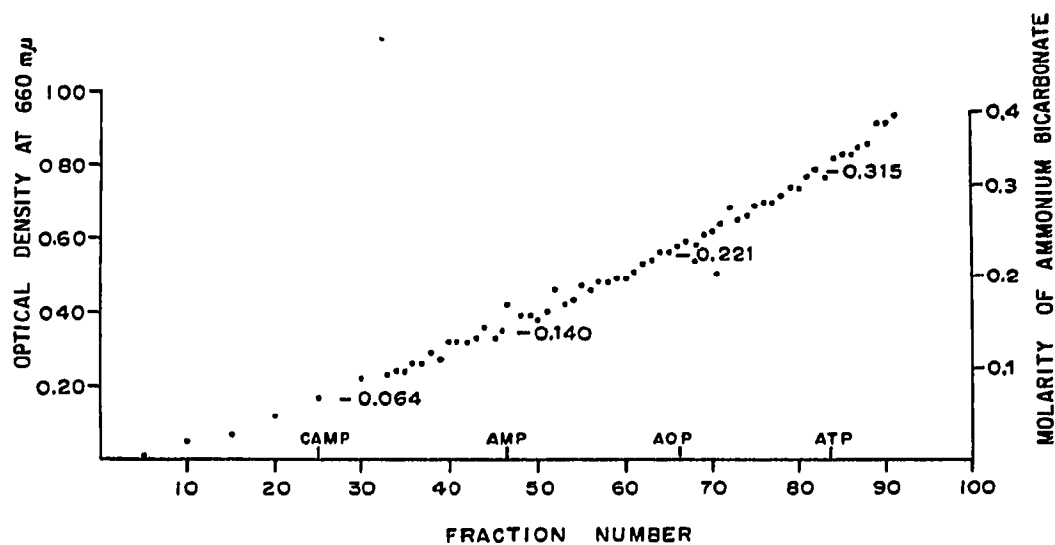
THE GRADIENT



Fig. 2. The shape of the gradient, determined by the use of Methylene Blue. The average center of each peak is indicated along with the predicted ammonium bicarbonate concentration.
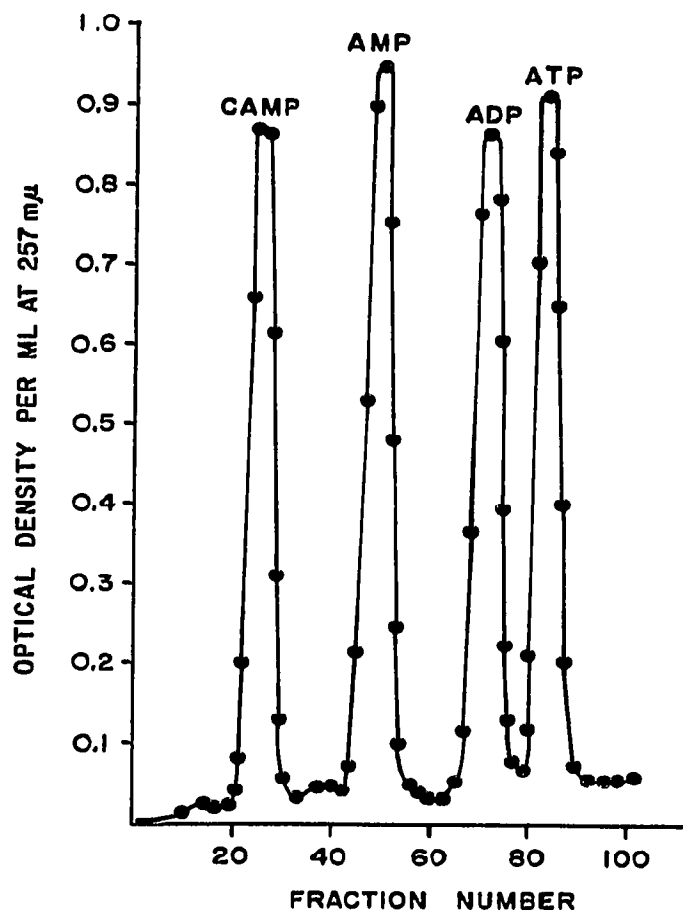


Fig. 3. A typical separation of four knowns added to the column.

recovered material, after subtraction of the column blank. The column blank was measured by doing a complete run without added nucleotides and measuring the optical density of the 100 fractions collected.

*Determination of the gradient.* Even though the shape of the gradient may be conveniently calculated[3,4], there is generally a deviation from the ideal due to variations in the shape of containers and volumes of connections. Therefore, we checked the shape of the gradient as follows: The gradient vessels were connected as in an experiment but the 0.4 $M$ ammonium bicarbonate solution was replaced by a solution of Methylene Blue in 0.4 $M$ ammonium bicarbonate having an optical density of a 1.0 at 660 m$\mu$. The optical density of each of the recovered fractions was then read at 660 m$\mu$ in a Coleman junior spectrophotometer and the results charted as shown in Fig. 2.

## Results

The results of a typical run where known amounts of known nucleotides were added to the column are shown plotted in Fig. 3. The nucleotides are eluted from the column in order of increasing acidity. It can be seen that there is a very clear separation between each of the knowns added, most especially CAMP. In the procedures that we have used the CAMP peak emerges completely in less than 1 h from the start. The columns described in the preceding section have accommodated up to 40 $\mu$moles of each of the four nucleotides and still managed a complete separation. We have also had very little difficulty in detecting a 2-$\mu$mole peak next to a 40-$\mu$mole peak by these procedures. In general, recoveries are very good, averaging 100 ± 1%. Typical recoveries are shown in Table I. In additional experiments we added varying amounts of standard solutions of each of the four nucleotides together and determined recoveries on each individual. There was a variation of as much as 15% in these procedures with ADP and ATP and sometimes cyclic AMP being low while AMP was generally high. Our suspicions that hydrolysis of the three more labile nucleotides was

TABLE I

RECOVERY OF KNOWN ADDITIONS TO COLUMNS

The figures in the first two columns represent total absorbancy at 257 m$\mu$

|      | Amount added | Recovery | %     |
|------|--------------|----------|-------|
| CAMP | 118 0        | 117 0    | 99 1  |
|      | 225.8        | 229.6    | 101 6 |
|      | 88.8         | 88.0     | 99 0  |
|      | 87 5         | 88 9     | 101 6 |
| AMP  | 117 3        | 117.0    | 99 7  |
| ADP  | 67.5         | 66 5     | 98 5  |
| ATP  | 109 5        | 110 0    | 99.5  |
|      | 203 3        | 204 7    | 100 6 |
|      | 97.8         | 90 9     | 97 9  |
|      | 95.5         | 95 5     | 100 1 |
|      | 103 5        | 101.3    | 97 8  |

causing this situation initially was substantiated by the fact that although individual recoveries might vary, the total recovery from total material added to the column was very close to 100%.

When we added freshly made standards one at a time to the column and determined the recoveries of the various peaks these suspicions were indeed verified.

*Discussion*

In general, methods for the separation of CAMP involve the use of more than one column whereas methods that separate the various adenine nucleotides by a single-column procedure make no provision for the isolation of CAMP. The procedures described in the preceding pages have been used by a number of persons with varying skills and training with equally good results. Indeed, it would seem that if this procedure were used in conjunction with a 260-m$\mu$ UV analyzer then it might be possible to obtain quantitative results directly from the optical density readings of the recorder.

It is necessary to repack a column before each analysis. However, since the Sephadex is fully expanded and stored in the chloride form with all fines removed it takes less than 15 min to empty out the used material, wash, and repack completely. We also used a number of variations in the shape, concentration, and quantities of the gradient. None of them were as satisfactory as the one shown in the preceding pages. There is also the possibility that faster pumping speed might considerably shorten the time for this separation. Although we have not tried this, there is no indication that this would not be a successful variation.

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1 L. S. BRADHAM AND D. W. WOOLLEY, *Biochim. Biophys. Acta*, 93 (1964) 475.
2 P. A. ÖCKERMAN, *Biochim. Biophys Acta*, 74 (1963) 588.
3 W. E. COHN AND F J BOLLUM, *Biochim. Biophys. Acta*, 48 (1961) 588.
4 R M. BOCK AND N S. LING, *Anal. Chem*, 26 (1954) 1543.
5 H SCHWAB, W. RIEMAN AND P. A. VAUGHN, *Anal. Chem.*, 29 (1957) 1357.