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SEPARATION OF NUCLEIC ACID HYDROLYSIS PRODUCTS, PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, RIBONUCLEIC ACID HYDROLYZATES, AND MIXTURES FROM NUCLEOTIDE SYNTHESES BY COLUMN CHROMATOGRAPHY ON AMBERLITE XAD-4

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SUMMARY

Amberlite XAD-4 resin has been studied as a support for liquid–solid column chromatography. By coating the resin with triethylammonium bicarbonate, a new and unique separation of nucleic acid components has been achieved. Separations are accomplished with a linear gradient of this buffer from 0.1 to 0.4 *M*. Separation occurs in the following order: inorganic phosphate, purine or pyrimidine bases, 5'-monophosphates, nucleosides and 5'-diphosphates or 5'-triphosphates; the 2'(3')-monophosphates are eluted after either the 5'-mono-, di- or triphosphates. The bases and nucleosides are separated in the order: cytosine, uracil, guanine and adenine. Inorganic phosphate and the nucleotides are eluted in the order: inorganic phosphate 5'-mono-, di- and tri-phosphates. Excellent separation of the 5'-monophosphates and the 2'(3')-monophosphates is now possible. In each series of 5'-mono-, di- and tri-phosphates or 2'(3')-monophosphates, the elution order is generally cytidine, uridine, guanosine and adenosine. By use of water instead of coating the resin with triethylammonium bicarbonate, the nucleotides and inorganic phosphate are found in the void volume; adenine is eluted very slowly, whereas adenosine is not eluted. Adenosine is eluted only with ethanol–water (1:3). The method is advantageous in that the recovery is quantitative, the buffer is easily removed, the capacity of the column is large (35 μmoles per gram of resin), flow-rates are high, the time required is short and separations of combinations of inorganic phosphate, bases, nucleosides and nucleotides are now possible that previously could not be accomplished.

INTRODUCTION

Amberlite XAD-4 is a cross-linked copolymer of styrene–divinylbenzene with a high level of divinylbenzene. The resin differs from XAD-2 in that it has a higher concentration of divinylbenzene, a larger surface area (750 m^2/g), a less porous structure (average pore diameter, 50 Å) and higher adsorptive capacity. A review of

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the application of these resins to the removal of toxic drugs in animal and human blood, to the purification and separation of steroids, carotenoids, amino acid mixtures, aromatic and aliphatic hydrocarbons and phenols has been made by Grieser and Pietrzyk[1]. Zaika[2] reported that nucleotides were not retained on XAD-2, and that bases and nucleosides were adsorbed and could be eluted with water at various pH values.

Existing column methods for the separation of purine and pyrimidine bases, nucleosides and nucleotides employ ion exchange, adsorption and partition as the basis of separation[3–15]. In adsorption and partition chromatography, fractionation is achieved by the use of solubility differences between the solid and liquid phases, or between two liquid phases, respectively. The ion-exchange methods utilize the pK_a values of the purine and pyrimidine bases, the nucleotides, the hydroxyl groups of the sugar moiety and/or differences in the borate-complexing potential of the sugar. Most recently, high-pressure liquid chromatography has been used effectively to separate biological compounds related to nucleic acids[16]. Although these methods are excellent, they suffer from the following limitations: first, one-step resolution of a mixture of purine and pyrimidine bases, nucleosides and nucleotides is not possible; second, except in a few cases, it is difficult and tedious to remove the buffers or the salts used for the separation.

Amberlite XAD-2 and XAD-4 possess, in common with charcoal, the property of high porosity and have been applied successfully to the desalting or to the extraction of certain compounds. The use of these resins has several advantages over traditional methods of solvent extraction or charcoal methods, including high recoveries and elimination of emulsions and filtration difficulties. However, the application of XAD-4 resin to the separation of polar compounds, which show little or no adsorption, has been limited. This problem has now been resolved by treatment of the copolymer with triethylammonium bicarbonate (TEA), followed by adsorption and separation of polar nucleosides and nucleotides by elution with the same buffer. The less polar nucleosides are eluted more rapidly than the polar nucleotides. Inorganic phosphate is eluted first. The nucleic acid compounds are quantitatively eluted in the following order: purine and pyrimidine bases, 5′-monophosphates, nucleosides, 5′-diphosphates and 5′-triphosphates. The 2′(3′)-monophosphates are eluted between the 5′-mono-, di- or triphosphates.

EXPERIMENTAL

Amberlite XAD-4 resin (20–50 mesh) was obtained from Rohm & Haas, Philadelphia, Pa., U.S.A. The resin was suspended in methanol and packed into a column. The resin bed was washed with methanol until the O.D. of the washings at 260 nm was less than 0.2. The washed resin was removed from the column and dried. The dry resin as then ground with a mortar and pestle and resin of 100–200 mesh was collected using 100- and 200-mesh sieves.

TEA buffer (0.5 *M*, pH 7.6) was prepared from triethylamine and carbon dioxide. All of the purine and pyrimidine bases, nucleosides and nucleotides used were of reagent-grade purity. Yeast ribonucleic acid (RNA) was obtained from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.). Intestinal alkaline phosphatase was obtained from Worthington Biochemical (Freehold, N.J., U.S.A.). Ultraviolet

(UV) and visible spectra were recorded on a Beckman DB spectrophotometer and on a Gilford Model 2400 spectrophotometer. Inorganic phosphate was assayed by the method of Ames[17].

Preparation of column and general chromatographic procedure

The bottom of each column was fitted with a small glass-wool plug. The resin (6 g, 100–200 mesh) was suspended in 100 ml of methanol. After 10 min the fine particles were decanted and the resin slurry was poured into a column (20 × 1 cm). The resin bed was washed with 500 ml of water in order to displace the methanol. Unless otherwise specified, the column was treated with 100 ml of 0.05 *M* TEA buffer. The sample (in less than 1.0 ml of water) was added to the column and the column was developed with a linear gradient of TEA (500 ml from 0.1 *M* to 0.4 *M*, pH 7.6). 5-ml or 10-ml fractions were collected at a flow-rate of 1.25 ml/min at room temperature using an automatic fraction collector. Columns were regenerated with 300 ml of methanol and 500 ml of water. Repeated use gave consistent results.

RESULTS AND DISCUSSION

Separation of nucleic acid components by water

In order to compare the elution characteristics of bases, nucleosides and nucleotides in water, the experimental procedure described was followed except that the column was not pre-treated with 0.05 *M* triethylamine buffer. The compounds used were adenosine 2′(3′)-monophosphate (2′(3′)-AMP), AMP, adenosine diphosphate (ADP), adenosine triphosphate (ATP), adenine and adenosine. A 1.0-ml solution of 2 μmoles of each component (in water) was added to the column and developed with 500 ml of water. The elution pattern of a series of adenine derivatives is shown in Fig. 1. The nucleotides were not adsorbed by the resin. Adenine was moderately adsorbed, while adenosine was retained so strongly that it was not eluted by 3 l of water. Adenosine was eluted with 200 ml of ethanol–water (1:3).

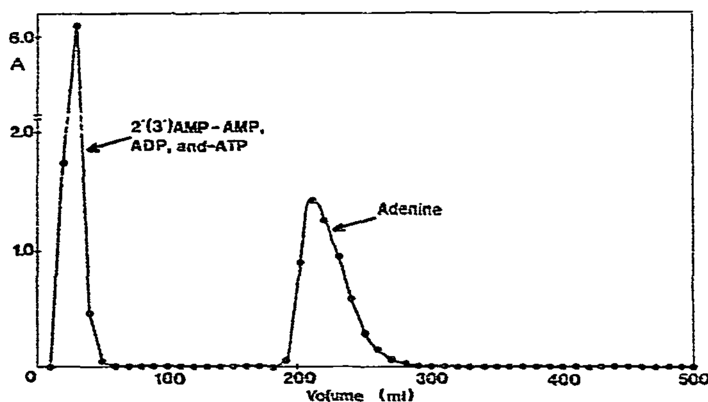

Fig. 1. Separation of nucleotides and adenine on Amberlite XAD-4 resin which had not been pre-treated with TEA buffer. Experimental conditions: 2 μmoles of each compound were added to XAD-4 resin (100–200 mesh) and then eluted with water; flow-rate, 1.25 ml/min; fractions collected, 10 ml; recovery, 95–100%. Absorbance measured at 260 nm.

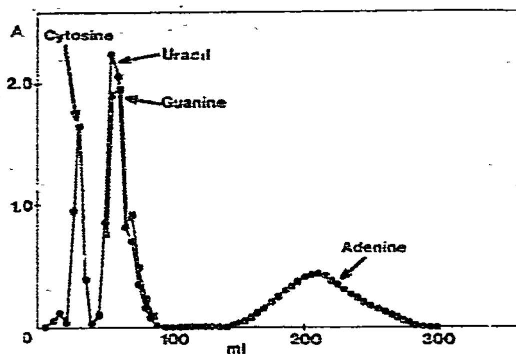

Fig. 2. Separation of the bases cytosine, uracil, guanine and adenine on Amberlite XAD-4 resin which had not been pre-treated with TEA buffer. Experimental conditions as in Fig. 1. 5-ml fractions were collected. Absorbance measured at 260 nm (●) or at 246 nm (■, guanine).

Fig. 2 shows the elution pattern of four bases with water. There was a tendency for pyrimidine bases to move faster than purine bases. Uracil and guanine were not readily separated. The more polar nucleotides had no affinity for the resin. The reason for the abnormal affinity of adenosine, which should be more polar than adenine, is not clear. However, this experimental observation is advantageous in that purine or pyrimidine bases and nucleosides can be separated by use of water and/or ethanol–water.

Separation of nucleic acid components by triethylammonium bicarbonate

Four bases (2 μmoles each, in less than 1.0 ml of water) were added to the column. The column (pre-treated with 0.05 *M* TEA buffer) was then developed with a linear gradient of the buffer (0.1 to 0.4 *M*; each of 250 ml). Fig. 3 shows the profile of the four bases. The volume of the buffer needed to elute cytosine, uracil and

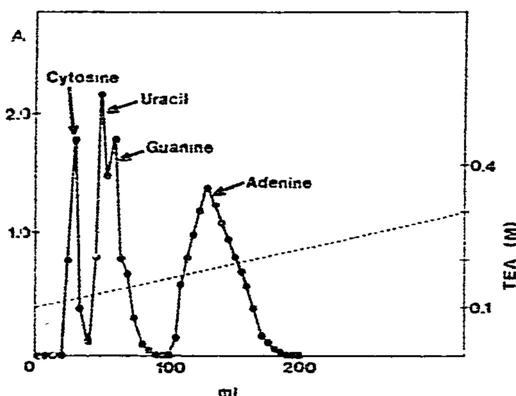

Fig. 3. Separation of purine and pyrimidine bases on Amberlite XAD-4 which was pre-treated with TEA buffer. Experimental conditions: 2 μmoles of each compound were added to 6 g of XAD-4 resin (100–200 mesh). The column (diameter, 1.0 cm) was pre-treated with 0.05 *M* TEA, and then developed with a linear gradient of from 0.1 *M* to 0.4 *M* TEA buffer (pH 7.6, each of 250 ml). 5-ml fractions were collected; flow-rate, 1.25 ml/min. Recovery, 98–100%. Absorbance measured at 260 nm.

guanine was essentially the same as that observed with water (Fig. 2), whereas adenine was eluted more rapidly. The separation of a mixture of four nucleosides using the same system is shown in Fig. 4. Adenosine was the most slowly eluted nucleoside. Adenosine was not eluted with water (see Fig. 1).

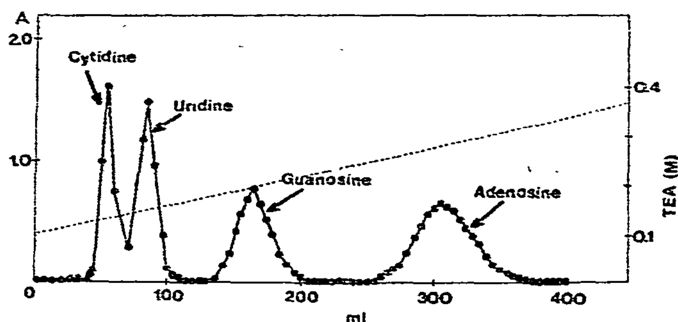

Fig. 4. Separation of purine and pyrimidine nucleosides on Amberlite XAD-4 which was pre-treated with TEA buffer. Experimental conditions as in Fig. 3.

In order to demonstrate the utility of this method to biological nucleotides, yeast RNA (5 mg) was hydrolyzed overnight in 0.1 *N* KOH at 37°. After neutralization with 70% perchloric acid at 0°, and centrifugation to remove potassium perchlorate, the supernatant was dried by means of a current of air. The hydrolyzate was taken up in 0.3 ml of a mixture of tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 8.0, 30 μmoles), $MgCl_2$ (5 μmoles) and 0.5 mg of chicken alkaline phosphatase, and incubated at 37° for 90 min. After heating for 3 min on a steam-bath, the mixture was poured on to a column and developed as described above. The resulting elution profile (Fig. 5) is identical with that of the standard mixture (Fig. 4). Each nucleoside was pure as determined by UV spectroscopy.

A similar procedure was applied to a series of nucleotides. As a test of the resolving power of the TEA-coated XAD-4 column, a mixture of inorganic phosphate, monophosphates, 5′-diphosphate and 5′-triphosphate was fractionated on the column. An example of the typical elution patterns obtained from a series of adenine

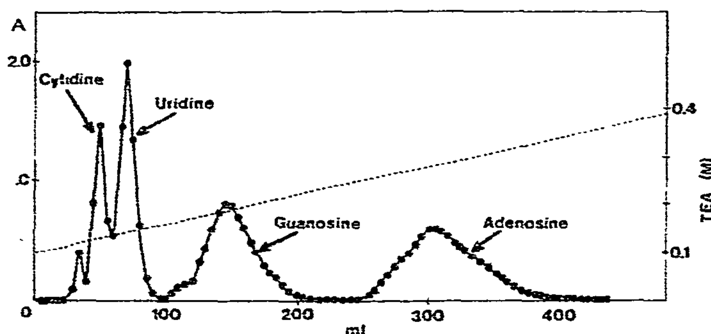

Fig. 5. Separation of yeast RNA hydrolyzate (5 mg) on Amberlite XAD-4 resin which was pre-treated with TEA buffer. Experimental as in Fig. 3.

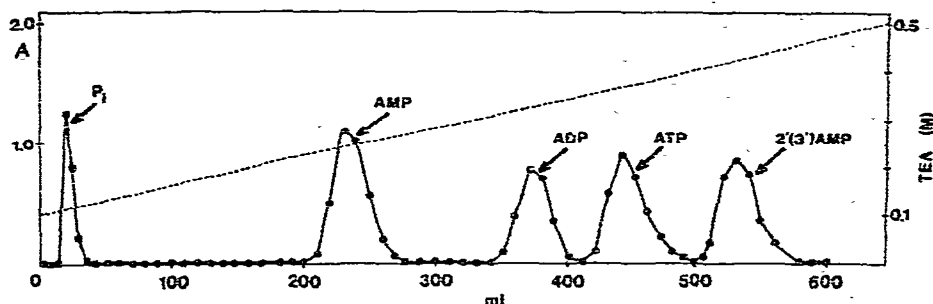

Fig. 6. Separation of inorganic phosphate and adenosine nucleotides on Amberlite XAD-4 resin which was pre-treated with TEA buffer. 10-ml fractions were collected. Absorbance measured at 820 nm (■) or at 260 nm (●). Other conditions as in Fig. 3.

derivatives is shown in Fig. 6. In a similar manner, a series of mixtures of uracil and guanine nucleotides were resolved by means of the same procedure. These results are summarized in Table I which gives the elution volumes (at the peak height for all of the bases, nucleosides and nucleotides).

TABLE I

ELUTION VOLUMES FOR PURINE AND PYRIMIDINE BASES, NUCLEOSIDES AND NUCLEOTIDES

Experimental conditions are as in Figs. 3 and 4; 2 μmoles of each compound were applied to the column. Separations were run in either the vertical or horizontal columns.

	Compound	*Elution volume (ml)*	*Compound*	*Elution volume (ml)*	*Compound*	*Elution volume (ml)*	*Compound*	*Elution volume (ml)*
Inorganic phosphate	P_i	20						
Purine or pyrimidine base	adenine	130	guanine	60	uracil	50	cytosine	30
5'-Monophosphate	AMP	230	GMP	120	UMP	80	CMP	50
Nucleoside	adenosine	305	guanosine	165	uridine	85	cytidine	55
5'-Diphosphate	ADP	370	GDP	240	UDP	180	CDP	120
5'-Triphosphate	ATP	440	GTP	380	UTP	320	CTP	220
2'(3')-Monophosphate	2'(3')-AMP	540	2'(3')-GMP	200	2'(3')-UMP	220	2'(3')-CMP	160

An additional example demonstrating the utility of this method is shown in Fig. 7. The nucleoside antibiotic formycin (26.7 mg, 100 μmoles) was converted into the 5'-monophosphate by the method of Yoshikawa *et al.*[18]. The reaction mixture contained inorganic phosphate, formycin 5'-monophosphate and unreacted formycin. Separation was achieved by use of the coated XAD-4 resin. The yield of formycin 5'-monophosphate was 50% and that of unreacted formycin was 50%. Recovery was 100%. It is apparent that all of the nucleotides which were tested are held on the resin and are eluted slowly in the presence of TEA in the order 5'-monophosphates, 5'-diphosphates and 5'-triphosphates. This indicates that slower elution of members of a group of components is related to the number of phosphate groups and to the location of the phosphate group on the ribose. The separations achieved by use of the

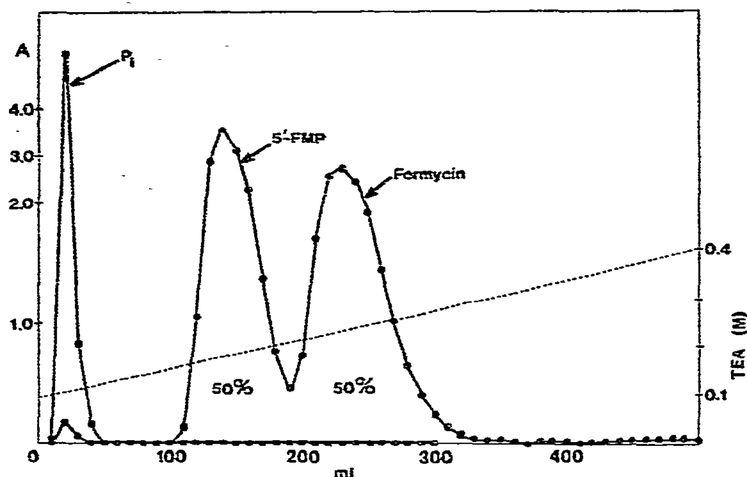

Fig. 7. Separation of inorganic phosphate, formycin 5'-monophosphate and formycin with Amberlite XAD-4 which was pre-treated on TEA buffer. 10-ml fractions were collected. Absorbance measured at 295 nm (●) or at 820 nm (■). Other conditions as in Fig. 3.

coated resin give elution profiles which are entirely different from those obtained on the uncoated resin. This might indicate that the 2'(3')-monophosphate has a steric configuration which is more favored for binding to the coated resin. In addition to the separation of nucleic acid components, inorganic phosphate was quickly displaced and well-separated from the nucleotides, nucleosides and bases. Thus, the use of this resin is a useful application for the desalting of bases, nucleosides and nucleotides. Recovery of all of the samples tested was quantitative. The use of a volatile buffer for elution greatly simplifies the separation of nucleic acid components and does not cause hydrolysis of the carbon–nitrogen bond of the purine nucleosides or nucleotides.

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