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The Separation and Estimation of Nucleotides

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A method is presented for the separation and estimation of the 5′-mono-, di- and triphosphates of adenosine, guanosine, cytidine and uridine together with uridine diphosphate glucose, uridine diphosphate acetylglucosamine, diphospho- and triphosphopyridine nucleotides. The nucleotides are separated by a combination of paper chromatography and ionophoresis, and an apparatus is described which allows the ionophoretic separation of substances with very small differences in mobility.

In the course of studies of the nucleotide content of different plant materials a method has been developed for the isolation of the acid-soluble nucleotides without interfering substances [1]. A quantitative separation of the mono-, di- and triphosphates of adenosine, guanosine, cytidine and uridine has been effected by means of a column of the strong base anion exchange resin Dowex-1. The resin was used in the formate form, and separation of the nucleotides was achieved by stepwise elution with formic acid solutions containing progressively increasing amounts of formate.

The use of ion exchange resins for chromatography is particularly well suited to the study of nucleoside derivatives because of the number and variety of charged groups which these compounds possess. Ion exchange chromatography has several great advantages. It is capable of handling both large and small quantities of material. The method is suitable for preparative work, great resolving power is attained and contaminants are of smaller importance than in paper chromatography and ionophoresis. As an analytical method this procedure suffers of slowness and of the disadvantage that considerable volumes of liquid may have to be handled. In order to get a more rapid and sufficiently accurate method for preliminary analysis, a procedure has been developed in this laboratory for the fractionation of nucleotides based upon chromatography together with ionophoresis on filter paper.

In spite of numerous trials with different solvent systems including those already reported in the literature together with a great number of other solvents, no method has so far been found to be entirely satisfactory for the separation of all the purine and pyrimidine nucleotides to be found in plant

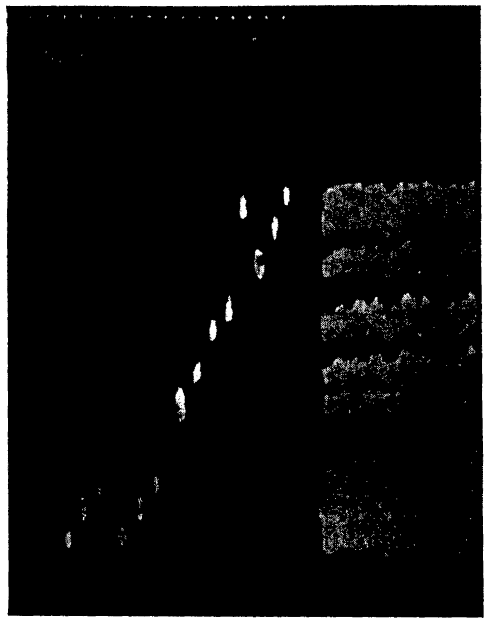

Fig. 1. One-dimensional chromatogram showing the group separation of a nucleotide mixture in comparison with the individual constituents. Solvent system: saturated ammonium sulphate solution-water-*iso*propanol (79:19:2); duration of the run: 35 h.

1) AMP;	4) DPN;	6) GMP;	9) CMP;	12) UMP;
2) ADP;	5) TPN;	7) GDP;	10) CDP;	13) UDP;
3) ATP;		8) GTP;	11) CTP;	14) UTP;
				15) UDPG;
				16) UDPAG;

materials. Two-dimensional paper chromatography has been used for the resolution of mixtures containing the 5′- mono-, di- and triphosphates of adenosine, guanosine, inosine and uridine giving adequate resolution of all the twelve components [2]. In the first direction, the chromatogram was developed with *n*-propanol-ammonia-water (60:30:10) and in the second direction with a solvent system consisting of saturated ammonium sulphate solution-water-*iso*propanol (79:19:2). The cytosine and uracil nucleotides are not separated from each other by means of these or any other solvent mixtures described in the literature. For the present purpose the use of the strongly alkaline first solvent system had the disadvantage of hydrolysing labile nucleotides like UDPG, UDPAG, DPN and TPN *. The ammonium sulphate mixture, originally introduced for the separation of the nucleotides obtained

* Abbreviations used: A, adenosine; G, guanosine; U, uridine; C, cytidine; MP, monophosphate; DP, diphosphate; TP, triphosphate; UDPG, uridine diphosphate glucose; UDPAG, uridine diphosphate acetylglucosamine; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

by hydrolysis of nucleic acids [3] and for the separation of adenosine and inosine polyphosphates [4] has, however, been retained from the previous method for obtaining a separation of the very complex nucleotide mixture into smaller groups.

The grade of filter paper most frequently used was Whatman No. 1, and when larger quantities were to be separated the thick Whatman No. 3 paper was used. For successful separation of the different groups it was necessary to use large sheets of paper (50 × 110 cm). Descending chromatography was used and the development was continued until the distance travelled by the solvent front was about 100 cm. The mixture to be analysed was applied to the paper in a series of small spots along a line across the paper, and in this way milligram amounts could be separated, which were even sufficient for the analysis of the weaker components. Emphasis must be laid on the necessity of always providing appropriate controls in chromatography with large papers thereby greatly decreasing the chances of erroneous deductions arising from unforseen variables, such as temperature changes etc.

Fig. 1 shows the fractionation of the actual nucleotides possible with this solvent system which, it will be noted, has a tendency to separate the compounds into four clear-cut bands. The components in the different bands are seen from the figure and are summarized in Table 1.

For quantitative estimations, each ultraviolet-absorbing band with the appropriate blank was cut out and eluted with 0.1 N hydrochloric acid. The optical densities of the extracts were read at the appropriate wavelengths and the total amount of nucleotides in the different bands could then be calculated. In order to concentrate the extracts sufficiently for further analysis, the substances were adsorbed on norite and eluted with small volumes of ethanol-ammonia.

The application of the method of paper ionophoresis to the separation of nucleotides has been extensively studied by various workers [5-8] but only a few papers are concerned with the separation of more than a few nucleotides. None of the techniques hitherto described has been found to give a satisfactory separation of all the nucleotides. With the following method a complete separation of all the nucleotides in the different groups was obtained, except for the uridine diphosphate sugar compounds.

Ionophoresis was carried out in sodium acetate buffer of pH 4.15 and of ionic strength 0.1. Higher and lower pH values resulted in overlapping of

Table 1. The nucleotides in the different groups obtained by paper chromatography as shown in Fig. 1.

Group	A	B	C	D
Compound	AMP ADP ATP DPN	TPN GMP	GDP GTP	CMP CDP CTP UMP UDP UTP UDPG UDPAG

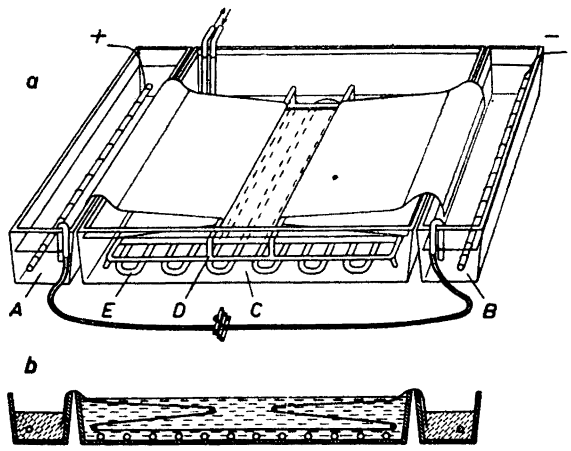

Fig. 2. Apparatus for paper ionophoresis, a) total view and b) cross section. A large sheet of filter paper, kept cool by immersion in the carbon tetrachloride vessel C (50 × 40 × 8 cm), is held in position by a glass frame D as shown in the cross section. The two ends of the paper dip into the electrode vessels A and B (50 × 10 × 8 cm). The liquid in the vessel C is cooled by running tap water in the coil E.

two or more of the cytosine and uracil nucleotides. Because of only small differences in mobility of several of the compounds it was necessary to use long travelling distances for getting sharp separations and therefore the apparatus shown in Fig. 2 has been used.

The equipment is a modification of the apparatus described by Markham and Smith [6], where the paper is kept cold by immersion in carbon tetrachloride. The apparatus consists of two vessels containing the buffer solutions and a third one containing carbon tetrachloride. Either carbon or platinum electrodes were used. Ionophoresis was carried out on large sheets of Whatman No.1 paper (40 × 110 cm) washed three times with N hydrochloric acid and finally with water for removing trace impurities. The samples to be analysed were applied to the paper at a line 15 cm from one end at right angles to the long axis of the paper. For getting compact spots the samples were applied as narrow bands instead of spots about 15 mm in length and about 3 cm from each other. The buffer was carefully brought up on either side of the samples through dipping in the desired electrolyte solution and the surplus moisture was removed by pressure between sheets of thick blotting paper. Then the paper, held in position by a frame made of glass, was immersed in the carbon tetrachloride vessel so that the two ends dipped into the electrode vessels, as shown in Fig. 2 b. The electrodes were connected to a d. c. power supply adjusted to 1 000 V giving a voltage gradient of about 10 V/cm. Under these conditions a run for 12 h gave a resolution of fractions A, B, C and D as shown in Fig. 3 in comparison with authentic nucleotides.

During the passage of the current through the long paper for several hours the carbon tetrachloride warmed up and to prevent this and to hold the

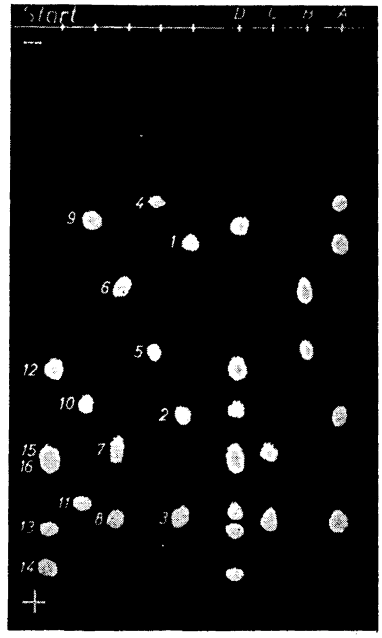

Fig. 3. Fractionation of the groups obtained in Fig. 1 by paper ionophoresis. Sodium acetate buffer of pH 4.15 and of ionic strength 0.1; 10 V/cm; 12 h. The mobility of the substances is compared with that of the authentic nucleotides and the numbers refer to the same substances as in Fig. 1.

temperature constant the liquid was cooled with tap water. The whole apparatus was placed under a cover which prevented the evaporation of the carbon tetrachloride.

When it is desirable to make a separation of a certain fraction on a large scale, it is advisable to apply the mixture as a line and if the substances to be separated have greatly differing mobilities a shorter paper can be used

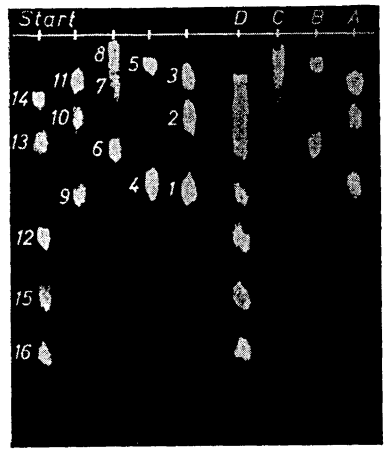

Fig. 4. One-dimensional chromatogram of the groups from Fig. 1 in 95 % ethanol-1 M ammonium acetate, pH 7.5 (75:30), 75 h. The reference substances have the same numbers as in Fig. 1.

to effect the fractionation. The length of the paper can be shortened by the use of other frames. The apparatus is suitable for two-dimensional ionophoresis and for a combination of ionophoresis and chromatography too, but these methods were not used in the present work.

When ionophoresis was completed, the paper was hung up to dry and then printed in ultraviolet light in the usual way. Then the spots containing the substances were located, cut out and eluted with 1 ml of 0.1 N hydrochloric acid. To allow for ultraviolet absorbing substances in the paper, densities were measured against the appropriate blanks. In this way the amounts of all the nucleotides could be calculated with the exception of UDPG and UDPAG, which were not separated from each other. The latter substances could be separated by chromatography in a solvent composed of 7.5 volumes of 95 % ethanol and 3 volumes of 1 M ammonium acetate solution of pH 7.5 [9]. Since the R_F values are rather low, it is advantageous to use the descending method and to cut notches in the papers at the bottom, from which the solvent is allowed to drip. Development for 75 h gave the separation shown in Fig. 4 in relation to authentic substances. Besides UDPG and UDPAG some other nucleotides were separated and another value of their concentration could be calculated. Several nucleotides, such as UDP, UTP, CDP and CTP or DPN and AMP, overlap in this solvent system. The guanosine polyphosphates form tails and are also badly separated.

Several mixtures of authentic nucleotides were analysed by the present procedure; the results of one of these analyses are summarized in Table 2.

Recoveries of 90—100 % were obtained regularly, the losses being mainly manipulational and no decomposition of any nucleotide could be detected. The method has been applied with success to the analysis of the acid-soluble nucleotide fraction from different plant materials.

EXPERIMENTAL

Paper chromatography

The group separation of the nucleotide mixture was performed in a large rectangular chromatographic chamber, 60 cm long, 30 cm wide and 115 cm high, which is large enough to handle up to 4 paper sheets, 50 × 110 cm. The atmosphere of the chamber must be saturated with the solvent mixture and therefore the walls of the chamber were lined with filter paper extending to the whole height of the chamber. Approximately 150 ml of the solvent mixture were poured into the tank, care being taken to wet the lining paper thoroughly. Before a run was started the papers were equilibrated with the solvent mixture for 8 h. When the solvent has run a convenient distance (in about 35 h) the paper was removed and dried at room temperature.

The nucleotides were located by making a contact print in monochromatic ultraviolet light of 254 mμ and then the bands were eluted and the quantitative evaluation of the amounts of material in each group performed as previously described. The nucleotides from each group were adsorbed on norite and eluted by repeated shaking with small volumes of an aqueous solution containing 50 % ethanol and 1 % ammonia. The elution was repeated in the cold until no more ultraviolet absorbing material was eluted, and the eluates were immediately adjusted to pH 7 with hydrochloric acid. In this way the concentrated solutions were also freed from salts which interfere with the ionophoretic separation.

Table 2. Analysis of a mixture of authentic nucleotides. The concentrations expressed in μmoles were calculated using the molecular extinction coefficients previously reported [1].

Compound	μmoles of nucleotide		Recovery %
	Calculated	Found	
AMP	2.39	2.28	95
ADP	3.02	3.05	101
ATP	3.24	3.08	95
DPN	2.89	2.72	94
TPN	2.16	2.10	97
GMP	2.77	2.74	99
GDP	2.74	2.74	100
GTP	2.95	2.77	94,
CMP	3.26	3.13	96
CDP	2.94	2.82	96
CTP	3.04	2.95	97
UMP	2.60	2.65	102
UDP	3.02	2.96	98
UTP	3.11	2.99	96
UDPG	2.68	2.55	95
UDPAG	2.40	2.33	97

The paper chromatographic separation in ethanol — 1 M ammonium acetate of pH 7.5 (75:30) was carried out with the standard size of paper (46 × 57 cm) and a conventional type of box was used. For quantitative estimations, the spots were cut out and eluted with 1.0 ml of 0.1 N hydrochloric acid. The optical densities of the extracts were measured in a 1.00 cm quartz cuvette in a Beckman Universal Spectrophotometer, Model DU, adapted for measuring small volumes.

Paper ionophoresis

Ionophoresis was carried out on Whatman No. 1 papers (40 × 110 cm) purified by washing three times with N hydrochloric acid and finally with water until the pH of the paper was about 7. The removal of interfering substances is particularly important in paper ionophoresis where the movement of nucleoside polyphosphates is otherwise severely retarded.

The material was applied to the dry paper at a cross line 15 cm from one edge; placing the substances in lines rather than circular spots gave much sharper separations. Sodium acetate buffer of pH 4.15 and of ionic strength 0.1 was used. Phosphate buffers were avoided because of the desirability of making phosphorus analyses on material eluted from the spots.

The paper was impregnated with buffer by dipping one edge of the paper into the buffer solution; when the buffer front has almost reached the samples, further movement

was arrested by dipping the other edge of the paper into the buffer and allowing this second front to meet the first. The excess moisture was removed and then the paper was placed on a glass frame and immersed into the vessel containing the carbon tetrachloride. After a run for 10 to 12 hours the paper was dried and then the nucleotides were revealed by taking an ultraviolet contact print. The quantitative estimations were performed as described above.

Materials

The nucleotides used were obtained from the Sigma Chemical Company. Stock solutions were first prepared of all the compounds and the composition of these solutions was carefully analysed by paper chromatography and ionophoresis. The mixture to be analysed was obtained by mixing carefully measured volumes of these stock solutions.

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