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The Separation of Nucleotides by Continuous Ionophoresis

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The separation of the adenine, guanine, cytosine and uracil 5'-ribonucleotides by continuous ionophoresis is described. The method is used for a quantitative separation and estimation of a complex nucleotide mixture.

The results obtained in a previous investigation [1] have demonstrated the value of paper ionophoresis for the separation and estimation of nucleotides. An apparatus was described which allows the ionophoretic separation of substances with very small differences in mobility. By a combination of paper chromatography and ionophoresis a complete separation 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 was obtained. The method to be described differs from the other one in that the mixture to be separated is continuously fed into the apparatus and the separated components can be continuously removed, so that it can be used for preparative purposes.

The method is based on the principle of passing a background electrolyte down a filter paper sheet by gravity. The mixture to be separated is applied at a prescribed intermediate position and passes along with the electrolyte as a narrow band. An electrical potential is applied across the filter paper curtain at right angles to the direction of flow and the various substances are deflected toward the anode or the cathode in accordance with their mobility behaviour in the electrical field.

The first successful apparatus using filter paper as a supporting medium for continuous ionophoresis was described by Grassmann and Hannig [2]. We have used a commercial model of their apparatus, which is available from the firm Bender and Hobein (Munich, Germany) under the name of "Elphor Va". This instrument has a free hanging curtain and is without cooling. A uniform field of potential is achieved by attaching the platinum wire electrodes to the vertical edges of the paper. In order to prevent diffusion of electrolysis products from the electrodes to the main body of the curtain, the electrodes are continually bathed with fresh buffer solution and are separated from the paper by a cellophan membrane. The rate of flow of the electrolyte down the paper and its composition must be kept constant. For this reason a supply of fresh

electrolyte is provided by allowing the paper to dip into a trough supplied with a device that maintains the electrolyte at a constant level. After passing through the paper the liquid is collected from 42 tongues cut in the paper along the lower edge. In this way the effluent is equally distributed over a number of fractions, each containing substances with mobilities within a certain small range. The mixture to be separated is injected by a mechanically operated syringe.

The degree of separation is determined by the electrophoretic mobility of the different components, the composition of the electrolyte, the voltage across the paper and the length of time that the substances are subjected to the field, which in turn depends on the grade of filter paper used. For a given flow rate, an increase in field intensity produces an improved resolution. When the current is too high, however, the water from the electrolyte may evaporate so rapidly that the downward flow is not sufficient to wash the paper, and in extreme cases electrolyte solids may be deposited, thus disrupting the steady state. A high flow rate enables larger amounts of material to be separated, but the separation of the different components increases the longer they remain on the paper. The final choice in the selection of experimental condition must be a compromise of the factors mentioned above.

The best separation of the nucleotides was obtained using an acetate buffer of pH 4.15, as has been shown earlier [1]. Since electrolytes of low ionic strength permit higher field intensities to be applied with relatively low current, it was found suitable to use a buffer of ionic strength 0.033. Many different types of filter paper with various degrees of porosity and thickness were tested and the best separation was obtained with Whatman No. 1 paper sheets, which were well washed with hydrochloric acid before use.

The maximum usable potential was found to be 320 volts. This voltage was applied across the platinum electrodes, and after about 6 h a steady state had been established. With this voltage a current of 17 mA was observed to flow constantly. The mechanically operated syringe was charged with the nucleotide mixture to be separated and fixed into position at a point in the upper part of the paper near the cathode and the synchronous motor was started. In order to obtain a quantitative recovery, it was necessary to collect fractions for about 20 h after all the sample had been applied to the paper.

Much useful information about the efficiency of the separation and to the location of the individual substances in the collecting tubes could be obtained from a study of the paper curtain itself. For this purpose the paper was carefully dried and then the different bands were located by making a contact print of the curtain in monochromatic ultraviolet light of 254 mμ.

The separation of mixtures of authentic adenine, guanine, cytosine and uracil nucleotides can be seen in Fig. 1, which shows the contact prints of the paper curtains from the separations. All the nucleotides tested could be separated with the exception of UDPG * and UDPAG, which were obtained as one fraction UDPX.

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

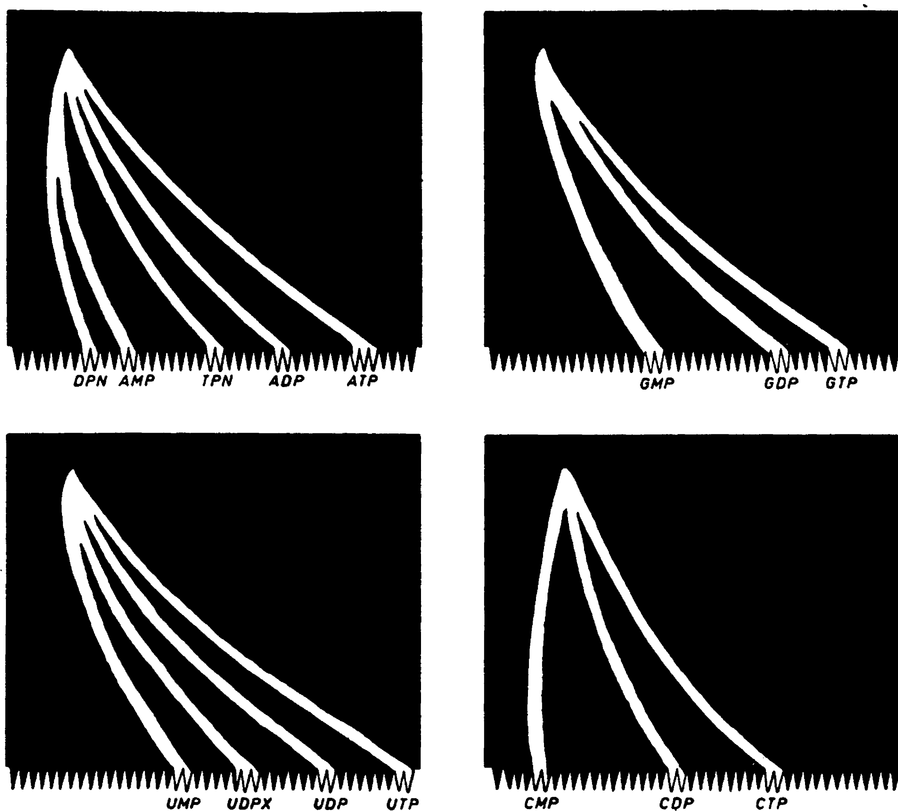

Fig. 1. Contact prints of the paper curtains from separations of groups of nucleotides by continuous ionophoresis. Conditions employed: Whatman No. 1 filter paper; sodium acetate buffer of ionic strength 0.033 and of pH 4.15; 320 V. The cathode was attached to the left edge of the paper curtain and the anode to the right one.

All the actual purine and pyrimidine nucleotides could be quantitatively separated after first fractionating the original mixture by means of paper chromatography [1] into the following groups: (1) AMP, ADP, ATP and DPN; (2) TPN and GMP; (3) GDP and GTP; and (4) CMP, CDP, CTP, UMP, UDP, UTP, UDPG and UDPAG. The different groups were eluted from the paper and concentrated sufficiently for further analysis by adsorption on norite and subsequent elution by ethanol-ammonia.

The concentrated solutions thus obtained could be separated into the different components by continuous ionophoresis. The separation of the different nucleotides was studied measuring the optical densities of the fractions collected from the individual tongues at 250, 260 and 270 mμ with the buffer as the blank. The fraction number and the absorbancy ratios E_{250}/E_{260} and E_{270}/E_{260} could be used for identification of the various nucleotides.

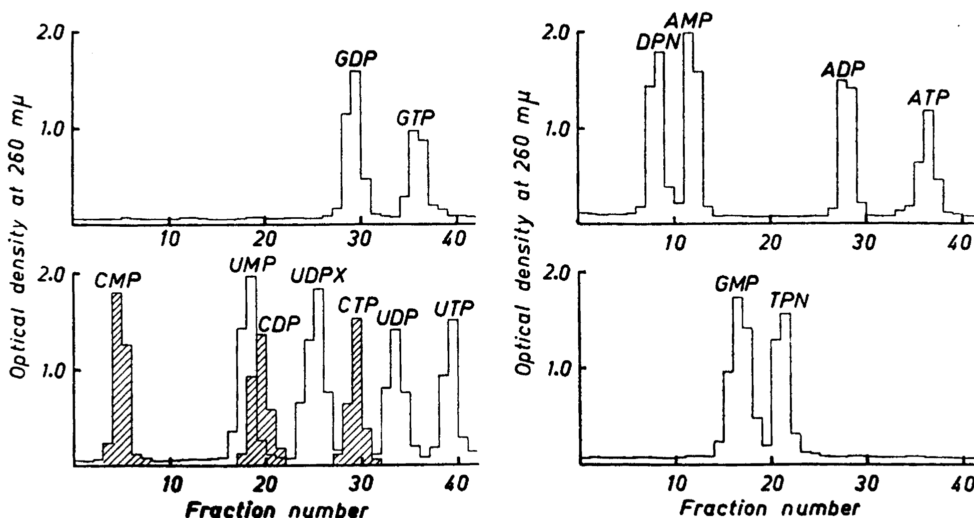

Fig. 2. Separations by continuous ionophoresis of the groups of nucleotides obtained after paper chromatographic fractionation. The same experimental conditions were used as described for Fig. 1. The fraction volumes were adjusted to 3.0 ml with buffer.

To obtain some quantitative measure of the separation efficiency test mixtures of known compositions were analysed by the combination of paper chromatography and continuous ionophoresis. Fig. 2 shows the diagrams obtained by the ionophoretic separation of the groups of nucleotides obtained after paper chromatographic fractionation.

The only components, which were not sufficiently separated, in addition to the uridine diphosphate sugar compounds, were UMP and CDP, but these

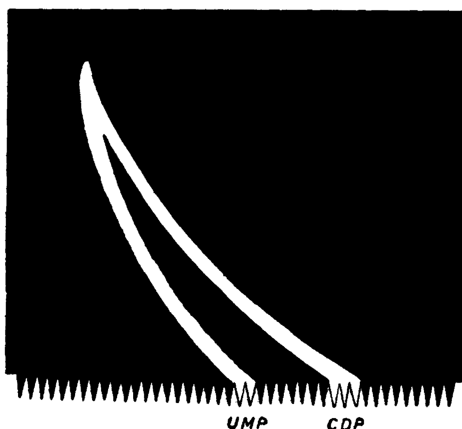

Fig. 3. Contact print of the paper curtain from the separation of UMP and CDP. The same experimental conditions as described for Fig. 1 were used besides that the buffer had a pH of 5.0 and the potential was 340 V.

Table 1. Analysis of a mixture of authentic nucleotides. The concentrations were calculated using the molecular extinction coefficients previously reported [3].

Compound	μmoles of nucleotide		Recovery %
	Calculated	Found	
AMP	3.16	3.13	99
ADP	2.80	2.58	92
ATP	2.32	2.16	93
DPN	2.56	2.25	88
TPN	2.36	2.17	92
GMP	5.48	5.15	94
GDP	3.92	3.80	97
GTP	2.72	2.45	90
CMP	6.52	6.25	96
CDP	6.04	5.75	95
CTP	5.20	4.95	95
UMP	4.96	5.00	101
UDP	4.00	3.84	96
UTP	3.68	3.24	88
UDPG	3.36	2.89	86
UDPAG	2.36	2.19	93

substances could easily be separated by paper chromatography or continuous ionophoresis with a buffer of other pH. Fig. 3 shows the separation of these substances with an acetate buffer of pH 5.0 and ionic strength 0.033.

Recoveries of 85—95 % were obtained, as can be seen from Table 1. Because of the number of components which are to be separated and the number end nature of the operations in these analyses, higher recoveries can hardly be expected. Yields of 95—100 % could be obtained for mixtures of nucleotides which could be separated directly on a single paper curtain. DPN was slightly decomposed during the separation but none of the other substances were found to be destroyed.

The earlier described apparatus [1] for one-dimensional ionophoresis with long travelling distances gives a sharper and more rapid separation of nucleotides than is obtainable by continuous ionophoresis. The presently described method is, however, more suitable for separations of larger amounts of material where the capacity of the other apparatus would be exceeded.

EXPERIMENTAL

Continuous ionophoresis. The apparatus consists of a framework holding a trough and the vertical electrode chambers. This frame is placed in a cemented glass case. The dimensions of the paper sheet are 47 × 47 cm, excluding the tongues. A 4 cm portion at the top dips into the trough and the vertical edges are pressed against the electrode chambers, but separated from them by a cellophan membrane. The washing of the electrodes and the constant level control of the solution in the trough is regulated by three Mariotte flasks.

Ionophoresis was carried out on Whatman No. 1 papers purified by washing three times with N hydrochloric acid and finally with water until the pH of the paper was about 7. An acetate buffer of ionic strength 0.033 and of pH 4.15 was used both as background electrolyte and to wash the electrodes. For the separation of UMP and CDP the buffer had a pH of 5.0.

The substance is applied by the insertion of the plunger of a glass syringe by means of a synchronous motor. This apparatus can give 0.020—0.900 ml per hour with different transmissions and syringes. The syringe is fitted with a glass tip joined to the syringe by polyvinyl chloride tubing of 0.6 mm bore. The point of application of the material is 8 cm below the lip of the trough and 9 cm from the cathode. The amount of each component applied per hour varied from 0.03 to 0.50 μmoles. At higher concentrations the zones became a little wider.

The different nucleotides could be identified and their concentrations determined from the optical densities of the fractions using the absorbancy ratios and molar absorbancies reported earlier [3]. The concentrations of the overlapping cytosine and uracil nucleotides could be approximately calculated from the absorbancy ratios.

When using exactly the same experimental conditions substances appeared in the same tubes for different analyses. With care the same paper curtain could be used for many separations.

Paper chromatography. The group separation of the nucleotides was performed as described earlier [1] on large sheets of Whatman No. 1 papers (50 × 110 cm) with the following solvent system: saturated ammonium sulphate-water-*iso*propanol (79:19:2).

The separation of the uridine diphosphate sugar compounds could be attained by paper 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. UMP and CDP could also be separated in this as well as in many other solvent systems.

Materials. The nucleotides used were obtained from the Sigma Chemical Company. The mixtures to be analysed were obtained by mixing measured volumes of stock solutions of known composition.

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