ELECTROPHORETIC METHOD FOR OBTAINING NUCLEOTIDES LABELLED WITH RADIOACTIVE CARBON

T. BORKOWSKI, H. BRZUSZKIEWICZ AND H. BERBEĆ

Biochemical Department, Medical Academy, Lublin (Poland)*

(Received February 5th, 1963)

INTRODUCTION

Ion-exchange chromatography is the method most frequently used for quantitative analysis of a mixture of nucleotides.

This method, introduced by COHN[1] in 1950, has been modified by numerous authors[2–6]. Recently NILSSON AND SJUNNESSON[7] described a method of separating nucleotides by means of chromatography on Ecteola-cellulose.

The analysis of a nucleotide mixture can also be carried out successfully by electrophoresis. This method, introduced by MARKHAM AND SMITH[8], makes it possible to obtain good separation of the four mononucleotides occurring in ribonucleic acid (RNA). TURBA, PELZER AND SCHUSTER[9] described a method of separating nucleotides by means of high-voltage electrophoresis; KLOUWEN[10] applied it to the separation of nucleotides extracted from tissues. Column electrophoresis[11] has also been employed.

All the methods mentioned above are used mainly for quantitative analysis of small amounts of nucleotide mixtures. The purpose of our present research was to develop a simple method of obtaining the four mononucleotides of RNA biosynthetically labelled with radioactive carbon.

EXPERIMENTAL

The four mononucleotides were separated by means of continuous electrophoresis on filter paper[12–14]. Electrophoresis was conducted on Whatman 3MM filter paper in an acid buffer solution at pH 3.5 (lithium acetate–acetic acid)[15]; the potential was 270 V, distributed over the whole sheet. The nucleotide mixture was applied continuously to the filter paper by means of a suitable device. The progress of the separation was checked with an ultraviolet lamp (Mineralight). The concentration of the nucleotides in the different test tubes was determined spectrophotometrically (a spectrophotometer of the Hilger type) in acid medium (pH 2.0) in 1 cm quartz cells.

Verification of the method

A sample of pure yeast RNA (British Drug Houses, Ltd., London) hydrolysed by the method of SCHMIDT AND TANNHAUSER[16] was used to determine the optimum con-

* Head: Prof. Dr. J. OPIEŃSKA-BLAUTH.

ditions for the separation of the four constituent mononucleotides. The RNA hydrolysate, acidified to pH 5.0, was introduced on to a column of suitably prepared activated charcoal[17]. The nucleotides were then eluted from the column with a solution of ammonia in alcohol. After evaporation to dryness, the eluate was transferred in 1 ml 0.1 *N* HCl to the dispensing vessel, and continuous electrophoresis carried out. The separation of the individual nucleotides on filter paper is shown in Fig. 1.

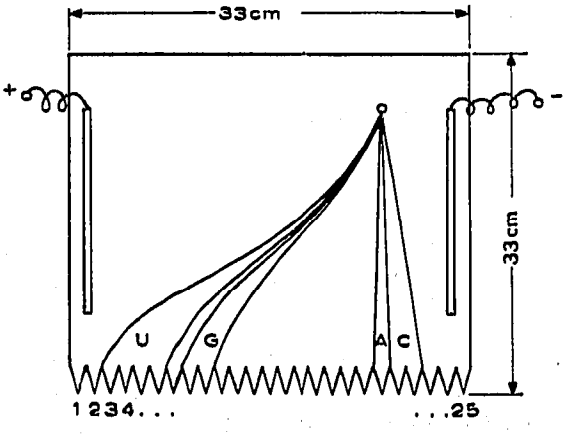

Fig. 1. Diagram of the electropherogram of the four mononucleotides. A = AMP; C = CMP; G = GMP; U = UMP.

The fractions collected in the test tubes were acidified to pH 2.0 and the absorption was determined for each of them at the following four wave lengths 250, 260, 275 and 280 mμ. The ratios of the absorption coefficients thus obtained are given in Table I, and were compared with those obtained by COHN[18] for pure nucleotides.

As can be seen from Table I, the absorption coefficient ratios calculated for the nucleotides in the present experiment differed slightly from those calculated for pure nucleotides. It was found that the buffer used in our experiments had a certain

TABLE I

ABSORPTION COEFFICIENTS OF THE FOUR MONONUCLEOTIDES

Nucleotide		*250/260*	*275/260*	*280/260*
AMP	T	0.85	0.43	0.22
	E	0.92	0.43	0.25
CMP	T	0.45	2.00	2.10
	E	0.52	1.65	1.67
GMP	T	0.96	0.69	0.67
	E	0.97	0.73	0.70
UMP	T	0.74	0.64	0.38
	E	0.86	0.59	0.36

T = theoretical values; E = experimental values.

TABLE II

ABSORPTION OF FILTER PAPER BACKGROUND

λ	250 mμ	260 mμ	275 mμ	280 mμ
$E \times 100/1$ ml	41.3	34.2	20.2	17.8

degree of absorption in the ultraviolet part of the spectrum. The absorption values due to the filter paper alone were determined for 1 ml of eluate at the four wave lengths and are listed in Table II.

Some absorption was found in the eluate from the filter paper alone, the greatest absorption being in the region of 250 mμ. This could account for the slight deviation of our absorption coefficients from the theoretical values.

For quantitative determination of the individual nucleotides we used the extinction value obtained at 260 mμ less absorption of the background at this same wave length. Alternatively the quantity of adenosine monophosphate (AMP), guanosine monophosphate (GMP) and uridine monophosphate (UMP) can be determined from the linear relationship between the difference in absorption at 260 and 280 mμ and concentration; this artifice eliminates the effect of the background absorption.

The value calculated in this way for cytidine monophosphate (CMP) was lower than the actual content of the nucleotide. We also determined the absorption curves of the individual nucleotides using the formula for the atomic extinction coefficient[19].

$$\varepsilon(P) = \frac{30.98 \times E}{c \times 1}.$$

The absorption curves obtained in the present experiment are shown in Fig. 2, where it can be seen that the characteristics of the curves are similar to those ob-

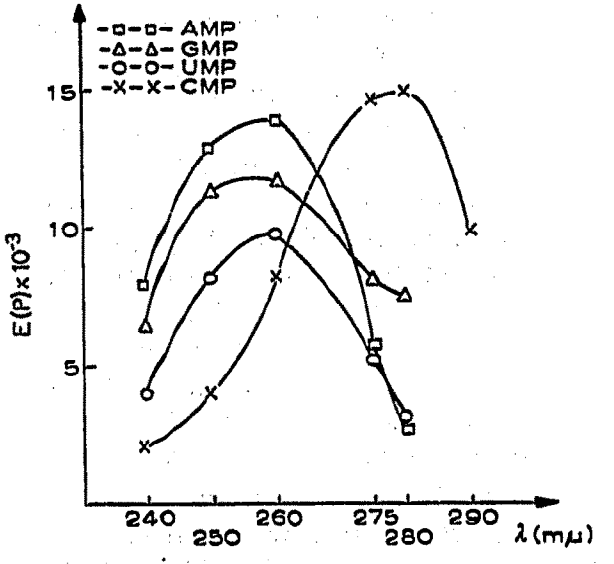

Fig. 2. Absorption curves of the four nucleotides separated by continuous electrophoresis. □——□ AMP; ×——× CMP; Δ——Δ GMP; O——O UMP.

tained for pure nucleotides. This confirms the efficacy of the separation and the purity of the nucleotides so obtained.

In view of the practical character of the present method, it seemed expedient to determine the losses resulting from the technical operations, or connected with the degree of separation of the different nucleotides on the filter paper. After applying 1.0 mg of nucleotide-P to the filter paper by the continuous method, 60 to 75 % of nucleotide-P can be obtained in the form of pure nucleotides. The extent of loss depends, apart from other factors, on the degree of separation of the bands of AMP and CMP. It can be seen from Fig. 1, that AMP and CMP occur close together on the filter paper, so that a "tongue" of the filter paper sometimes furnishes a mixture of both nucleotides.

With the present method, provided that a suitable concentration of the mixture is applied to the filter paper, 6 to 10 μmoles of each mononucleotide can be obtained after 18 h electrophoresis.

Separation of nucleotides biosynthetically labelled with ^{14}C

Having established the general principles of the method for separation we proceeded to prepare ^{14}C-labelled nucleotides by biosynthesis. The method used was based on that of DOWNING AND SCHWEIGERT[20] AND MANTSAVINOS AND CANELLAKIS[21] for the production of desoxyribonucleotides. In our experiments *E. coli* was used, which when incubated in the presence of $NaH^{14}CO_3$, incorporates ^{14}C into the nucleic acids. The initial radioactive compound was $Ca^{14}CO_3$, which was converted to sodium bicarbonate in the Van Slyke–Folch apparatus.

500 μC of $Ca^{14}CO_3$ was used for a single experiment. The $NaH^{14}CO_3$ was added to the incubation medium, simultaneously with a suspension of *E. coli*, which had been passed through a synthetic medium in the presence of non-radioactive sodium bicarbonate. The synthetic incubation medium used in the labelling process contained glucose, sodium citrate and radioactive sodium bicarbonate as sources of carbon. Incubation was carried on for 18 h at 37° in an apparatus which prevented radioactive carbon dioxide from escaping into the atmosphere. Under these conditions intense multiplication of the bacterial cells takes place, and radioactive carbon is incorporated into the cellular components. Growth was stopped by heating the bacteria suspension to 50°, and the culture was aerated at the same temperature in order to remove the remaining carbon dioxide from the medium.

The bacterial mass was centrifuged, washed twice with cold 0.9 % NaCl, and then lyophilized. The dry powder was extracted twice with anhydrous ethyl ether at 0°, followed by two extractions with 0.6 *M* $HClO_4$ at 0°, and then washed with water. The sediment was extracted twice with boiling ethanol, and twice with a mixture of methanol and chloroform. The powder was then subjected to alkaline hydrolysis. The nucleoside-3'-monophosphates so obtained were purified on a column of activated charcoal and then separated by means of continuous electrophoresis according to the method already described.

The radioactivity of the different fractions was determined, during the successive stages of the experiment. This was done by counting a dried layer of the sample with a Geiger-Müller counter having a mica window (1.4 mg/cm^2); the counter was placed 10 mm from the sample. The concentration of RNA was determined by means of a spectrophotometer, the calculations being carried with out the coefficients given by

TSANIEV[22]. The distribution of the isotope label over the different stages of the experiment is shown in Table III where it can be seen that the radioactivity of the non-purified RNA preparation is much higher than that obtained after purification on a charcoal column. The radioactivity of each of the isolated mononucleotides approaches that of RNA, the isotope label being distributed evenly over all the four mononucleotides.

TABLE III

DISTRIBUTION OF RADIOTRACER IN THE RADIOACTIVE NUCLEOTIDES OBTAINED BY BIOSYNTHESIS

Analysed compound	*Quantity of analysed compound in μg P*	*Activity C/min/μg P*	*Specific activity C/min/μmole*
RNA non purified	828.0	975	—
RNA after purification	746.0	570	—
AMP	101.5	510	15,800
CMP	107.4	510	15,800
UMP	172.2	516	16,000
GMP	168.6	480	14,600
Adenine	45.0 μg	—	14,700
Cytosine	63.0 μg	—	14,300
Uracil	52.0 μg	—	15,200
Guanine	41.8 μg	—	14,300

In order to discover the position of the radioactive carbon in the nucleotides obtained in the present experiment the pure preparations were hydrolysed. Purine nucleotides were hydrolysed in 1 *N* HCl for 1 h at 100°.

After vacuum drying at a low temperature, the hydrolysate residue was dissolved in water and applied to the filter paper. Pyrimidine nucleotides were hydrolysed in 60 %: $HClO_4$ for 1 h at 100°. The perchloric acid was removed by cold precipitation in the form of $KClO_4$. The pure hydrolysate was condensed by lyophilization, and then dissolved in a small quantity of water, and applied to the filter paper.

Descending chromatography was carried out in the isopropanol–HCl[23] or in butanol–ammonia[8] for 24 h. The ribose spots on the chromatogram were localized by the aniline test[24], or by the silver nitrate test[25]. Spots of purine and pyrimidine bases were observed in ultraviolet light, a suitable filter being used in the lamp. In addition, the radioactivity was determined by direct measurement on the chromatograms.

The distribution of the isotope on the chromatograms is shown in Fig. 3. The whole of the activity was found to be in the purine base, whereas the activity in the spot where ribose is located on the chromatogram was equal to that of the background. The purine and pyrimidine bases separated chromatographically from the radioactive nucleotides were eluted from the chromatograms with 0.1 *N* HCl.

The concentration of the individual bases in the eluates was determined spectrophotometrically, and their activity measured. The specific activities found for the individual bases are listed in Table III; they hardly differ from those of the nucleotides from which the bases had been obtained. It can be concluded that the radioactive carbon is only incorporated into the base part of the nucleotide.

In the experiments in which 500 μC of sodium bicarbonate was added to every 700 ml of the incubation medium, the isolated nucleotides differed only slightly

from one another in their specific activity. In one experiment, in which 600 μC $NaH^{14}CO_3$ was used, the activity of the obtained nucleotides was proportionally higher.

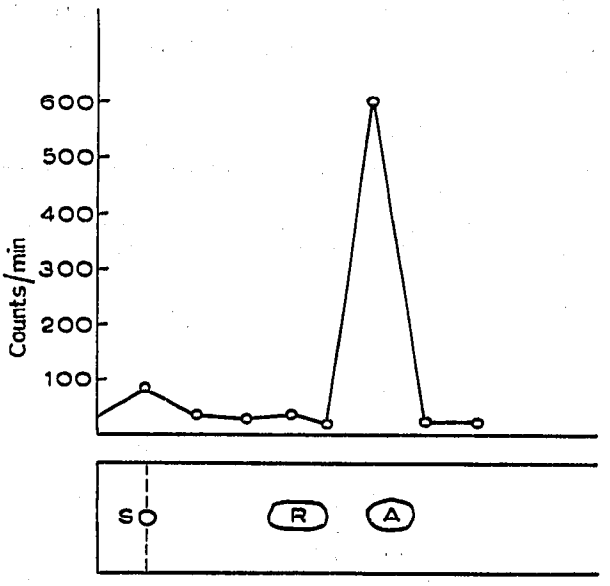

Fig. 3. Distribution of the radiotracer in the hydrolysate of adenylic acid as revealed by paper chromatography. S = starting point; R = ribose; A = adenine.

DISCUSSION

Unlike most methods mentioned in the introduction, our method of separating the four mononucleotides is characterized by great simplicity. MANTSAVINOS AND CANELLAKIS, using chromatography on a column with Dowex 1, obtained from 400 mg of lipid-free bacteria powder, 8 to 13 μmoles of each of the desoxynucleotides of DNA. Our method also makes it possible to obtain 6 to 10 μmoles of each ribonucleotide. Detailed analysis demonstrated that the nucleotides obtained by our method were of high purity and the losses resulting from all operations connected with the electrophoresis did not exceed 30 %.

The analysis of the activity of the individual fractions obtained in our studies on ^{14}C-labelled nucleotides confirmed the fact that ^{14}C is incorporated into various chemical compounds. Both the fraction of acid-soluble compounds and the lipid fraction of the culture of *E. coli* showed a high degree of activity. An equally high activity was found in a neutralized alkaline hydrolysate of bacterial RNA. After purification of the hydrolysate on a charcoal column, the activity per μg of nucleotide-P decreased considerably in comparison with the corresponding value in a non-purified alkaline hydrolysate. On the other hand, the activity of the individual nucleotides did not differ from the activity of the sum of nucleotides contained in the RNA purified on a charcoal column. It is known from the work of TSANIEV AND MARKOV[20], and FLECK AND MUNRO[26] that when biological material is subjected to alkaline hydrolysis, not only nucleotides, but also products of protein decomposition, such as peptides and phosphopeptides pass into the hydrolysate. A comparison of the values, obtained in the present research, for the activity of RNA before and after purification indicates the presence in the alkaline hydrolysate of non-

nucleotide products of high activity. These findings might confirm the observation that in the case of bacteria, $^{14}CO_2$ is also incorporated into amino acids. It was of more interest to us to investigate to what degree the bases and the ribose in the nucleotides had been labelled.

Our experiments demonstrated that the whole of the radiotracer is located in the purine and pyrimidine bases as the bases obtained from nucleotides showed the same specific activity as the nucleotides themselves.

In the majority of studies carried out with radioactive carbon, a flow counter is used to measure the activity as it is a better detection of soft β-rays; however, a Geiger-Müller counter with a mica window was used in the present experiments. Thus the specific activity found by us for the individual nucleotides represents a high degree of incorporation of isotope, as the efficiency of the counter is low for ^{14}C It would seem that the ^{14}C-labelled nucleotides obtained in our experiments could be used for investigating the synthesis of RNA.

SUMMARY

The conditions for separating AMP, GMP, CMP and UMP by means of continuous electrophoresis on Whatman 3 MM filter paper in acid buffer at pH 3.5 are described. The ribunucleotides of RNA obtained in this way were tested with regard to their purity.

The method can be used for the production of the four mononucleotides on a laboratory scale. Good results were obtained for the production of nucleotides from biosynthetically labelled RNA of *E. coli* grown on a synthetic medium in the presence of $^{14}CO_2$.

The radioactive mononucleotides were found to be labelled in the purine and pyrimidine bases only. The specific activity of the four mononucleotides was similar.

REFERENCES

1 W. E. Cohn, *J. Am. Chem. Soc.*, 72 (1950) 1471.
2 W. E. Cohn, *J. Biol. Chem.*, 203 (1953) 319.
3 R. Bergkvist and A. Deutsch, *Acta Chem. Scand.*, 8 (1954) 1877.
4 R. B. Hulbert, H. Schmitz, A. F. Brumen and V. R. Potter, *J. Biol. Chem.*, 209 (1954) 23.
5 H. G. Pontis and N. L. Blumson, *Biochim. Biophys. Acta*, 27 (1958) 618.
6 R. K. Crane and F. Lipmann, *J. Biol. Chem.*, 201 (1953) 235.
7 R. Nilsson and M. Sjunnesson, *Acta Chem. Scand.*, 15 (1961) 1017.
8 R. Markham and J. D. Smith, *Biochem. J.*, 45 (1949) 294.
9 F. Turba, H. Pelzer and H. Schuster, *Z. Physiol. Chem.*, 296 (1954) 97.
10 H. M. Klouwen, *J. Chromatog.*, 7 (1962) 316.
11 N. Frontali, *Acta Chem. Scand.*, 13 (1959) 390.
12 H. Svenson and L. Bratson, *Arkiv Kemi, Mineral. Geol.*, 1 (1949) 401.
13 W. Grassman and K. Hannig, *Naturwiss.*, 37 (1950) 397.
14 W. Grassman and K. Hannig, *Z. Physiol. Chem.*, 292 (1953) 32.
15 N. Virmeaux, personal communication.
16 G. Schmidt and S. J. Tannhauser, *J. Biol. Chem.*, 161 (1945) 83.
17 J. R. Bertlett, *J. Biol. Chem.*, 234 (1959) 465.
18 W. E. Cohn, in S. P. Colowick and N. O. Kaplan (Editors), *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 724.
19 E. Chargaff and S. Zamenhof, *J. Biol. Chem.*, 173 (1948) 327.
20 M. Downing and B. S. Schweigert, *J. Biol. Chem.*, 220 (1956) 513.
21 R. Mantsavinos and E. S. Canellakis, *J. Biol. Chem.*, 234 (1959) 628.
22 R. Tsaniev and G. G. Markov, *Biochim. Biophys. Acta*, 42 (1960) 442.
23 G. R. Wyatt, *Biochem. J.*, 48 (1951) 584.
24 S. M. Partridge, *Nature*, 164 (1949) 443.
25 W. Z. Travelyan, O. D. Proctor and J. S. Harrison, *Nature*, 166, (1950) 444.
26 A. Fleck and H. N. Munro, *Biochim. Biophys. Acta*, 55 (1962) 571.