Separation of Purines and Pyrimidines by Paper Electrophoresis

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An accurate and fairly rapid method has been worked out for the quantitative determination of purine and pyrimidine bases by paper electrophoresis.

Paper electrophoresis does not seem to have been widely used for the quantitative determination of purines and pyrimidines in nucleic acids hydrolysates. Dimroth *et al.*¹ have reported the separation of adenine from the mixture of the other purines and pyrimidines at pH 3.2, in acetate buffer. Burma² reported a successful separation of the four bases in nucleic acid hydrolysates at pH 2 (citrate-phosphate buffer), with 220 V, during 8 hours. Karyone and Inoue³ also reported a separation of purines and pyrimidines, but no detailed data on their work are available.

In the following, a procedure for the quantitative estimation of purines and pyrimidines by paper electrophoresis is described. As it will be shown, it has some advantages, especially over paper chromatography, but also over other electrophoretic procedures, since it is both more rapid and more accurate, even with very little material.

Electrophoretic separation of the bases from a nucleic acid hydrolysate can only be performed at acidic pH's, because the low solubility of A and G** leads to a considerable tailing at neutral and alkali pH's. Separation is possible at pH 3.8 (order of travelling towards the cathode: C, A, T+U, G) and at pH 3 (order of travelling: C, A, G, T+U). At intermediate pH values, a separation between G and U+T is not possible; at pH lower than 3, A and C tend to travel together.

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** Abbreviations: A, Adenine; C, Cytosine; G, Guanine; T, Thymine; U, Uracil; WS, Levy and Snellbaker's substance 4; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

pH 3 was chosen, because at pH 3.8 a certain tailing of G was often observed. Table 1 reports the p*K*'s of dissociation of the amino groups of the bases and their percent dissociation at pH 3.

Table 1. Dissociation constants of the amino groups of the bases and their percent dissociation at pH 3.

	p <i>K</i> 's of the amino groups ⁵	Percent dissociation at pH 3
Cytosine	4.6	98
Adenine	4.1	92.5
Guanine	3.2	61.5
Thymine	—	—
Uracil	—	—

The relative electrophoretic mobilities, at pH 3, agree fairly well with those calculated from the figures of Table 1.

Although not tested, it is to be expected that 5-methylcytosine should travel with C.

When a hydrolysate of DNA (Wyatt method ⁶) is separated by the method described here, a white-fluorescent spot travels with G. This contaminating substance is likely to be identified with the WS (white spot) described by Levy and Snellbaker ⁴, since the dissociation constants of its groups are very close to those of guanine. Moreover, the UV spectrum of G + WS eluted after paper electrophoresis, corresponds to that of a mixture of G + WS (*e. g.*, the E_{280}/E_{260} ratios are: for G⁷, 0.84; for WS⁴, about 0.40, and for the eluted mixed spot: 0.74). Since it has been shown ⁸ that WS is likely formed from G during the hydrolysis of DNA according to Wyatt, eluting and determining the WS together with its parent substance reduces this source of error.

A high electroosmotic flow was found, when Whatman paper No. 1 was used. This is an advantage, as it helps to avoid contamination of the T and U spots with the dark-coloured material formed during the hydrolysis, which remains at the starting point.

Citrate buffer was chosen, because of its low UV extinction.

PROCEDURE

DNA (prepared from calf thymus according to Kay *et al.*¹¹) was hydrolysed according to Wyatt ⁶. The hydrolysate was brought to dryness, and taken up in enough 1 N HCl, to have 5 to 50 μ g of each base in 5 μ l.

A humid chamber horizontal electrophoresis apparatus with Whatman No. 1 paper strips (10 cm wide and 42 cm long) and citrate-HCl buffer, pH 3 (Sørensen), diluted to an ionic strength of 0.02 ($\kappa^{-1} = 0.175 \times 10^4$ ohms at 21° C) was used.

Samples of 5 μ l were applied at the starting line, in a narrow zone, after the paper strips had been equilibrated in the apparatus. A part of the paper

Fig. 1. Separation of purines and pyrimidines from a DNA hydrolysate by paper electrophoresis (citrate-HCl buffer, pH 3, μ 0.02, 440 V, during 3 hours on Whatman No.1).

strip was left free for the blanks. A voltage of 440 V, 7–8 mA, was applied during about 3 h at room temperature. The paper was then dried in an oven at 100° C for 5 min. The spots were located with a UV television set⁹ or according to Holiday and Johnson¹⁰. The spots were very compact, without any tailing, in all runs (see Fig. 1).

The spots were eluted overnight with 3.5 ml (or 1.2 ml; in this case, semi-micro cuvettes were used for UV readings) 0.1 N HCl (1 N HCl for G). Readings were taken against water at 250, 260, 280 and 290 $m\mu$. A Beckman DU spectrophotometer was used; the values were corrected for the blanks of the paper (which ranged usually between 0.010 and 0.030).

The comparison between the ratios of the optical densities at these wavelengths, and those reported by Volkin and Cohn⁷ showed excellent agreement, except in the case of G, for the reasons reported above. The extinction coefficients at 260 (for A, G and T) or 280 $m\mu$ (for C) reported by Volkin and Cohn⁷ were used.

As an example of determination of purine and pyrimidine composition of DNA, the following figures are reported (Table 2), dealing with calf thymus DNA. Ten separate runs were made from a single hydrolysate; each spot contained 5 to 10 μ g of each base.

Table 2. Composition of calf thymus DNA, as determined by paper electrophoresis. The values ($\bar{x}$) are expressed as moles per 100 moles of total bases recovered, and represent the averages of ten determinations, on a single hydrolysate. The standard deviation (σ) are expressed as percent of $\bar{x}$.

	$\bar{x}$	σ
Adenine	29.0	$\pm 1.67 \%$
Thymine	28.5	$\pm 2.64 \%$
Guanine		
(+ WS)	21.2	$\pm 3.75 \%$
Cytosine	21.3	$\pm 4.75 \%$
(+ 5-methyl-cyt.)		

As it can be seen, the standard deviations σ in this method are very low. The $\frac{A + T}{G + C + 5MeC}$ ratio is 1.35. It agrees fairly well with the figures reported in the literature: 1.20–1.22¹²; 1.27¹³; 1.28¹⁴; 1.37¹⁵.

For quantitative purposes, the method described above seems to have some advantages over other methods:

1) It is quite accurate: in the author's hands no other method tested has given standard deviations $< 5\%$, when as little as 5—10 μg of each base were used. The method described here can therefore be recommended even when little material is available, as in column chromatography of DNA.

2) Levy and Snellbaker's substance (WS)⁴ is determined with its parent substance, *i. e.* guanine⁸. In this way, too low guanine values, as well as too high values of other bases (contaminated by WS) are avoided.

3) Because of the high electroosmotic flow, contamination with the insoluble material formed during the hydrolysis (and not moving from the starting line) is also avoided.

4) It is quite rapid: a 3 hours' run gives a complete separation.

5) This procedure can also be used to separate the four bases (U, G, A, C) obtained from a strong acid hydrolysis of RNA.

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