Journal of Chromatography, 128 (1976) 304–308

CHROM. 9314

Note

Two-dimensional combination of the hydrogen and the ammonium forms of a cation-exchange layer for the separation of minor nucleic acid bases*

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(First received March 17th, 1976; revised manuscript received May 5th, 1976)

Two-dimensional ion-exchange paper chromatography or thin-layer chromatography (TLC) has successfully been applied to the resolution of complex mixtures of a variety of compounds (see, for example, refs. 1–3). The ion exchanger has been used in identical form, generally a salt form in both dimensions, although it may be partly transformed into another salt form during developments, when ions different from the counter-ion are used for displacement. We report here a separation in which the hydrogen form is used in one direction and a salt form in the second direction for the separation of nineteen purine and pyrimidine bases.

The technique was evolved during studies on the separation of nucleic acid constituents by cation-exchange TLC; in these studies, the layer was used in either the hydrogen or the salt form[4–9].

EXPERIMENTAL

All chemicals were of reagent grade. Purine and pyrimidine bases were commercial products (Sigma, St. Louis, Mo., U.S.A.) and their purities were checked by paper chromatography and UV absorption[10].

The TLC was performed on chromatoplates (20 × 20 cm) coated with a strong cation-exchange resin, *viz.*, Polygram Ionex 25 SA (Macherey, Nagel and Co., Düren, G.F.R.) and Fixion 50-X8 (Chinoin, Nagytétény, Hungary). Commercial chromatoplates in the sodium form were converted into the hydrogen form by developing them with 1.0 *N* hydrochloric acid and then with deionized water for 16 h with each solvent according to the usual continuous ascending technique[7]. The mixture of compounds to be separated (about 1 μg of each) was applied as a spot 3 cm from both the lower edge and the left-hand edge of a chromatoplate.

After development in 1.0 *N* hydrochloric acid (System I), the layer was dried by a stream of cold air for 20–30 min. The chromatoplate was fixed to the cover of a petri dish (diameter 29 cm) by moistening its uncoated side, and the cover was then placed on the dish, which contained concentrated aqueous ammonia. After exposure

* Part VII in the series "Separation of nucleic acid bases, nucleosides and nucleotides on strong cation-exchange thin layers". For earlier parts, see refs. 4–9.

for 10 min to the ammonia vapour, the chromatoplate was removed, and surplus ammonia was evaporated by a stream of cold air. The capacity of the layer was determined as described by Helfferich[11] before and after the treatment with ammonia. Before development in the second direction, the area above the spot having the highest R_F value of the first direction was cut off and discarded. For development in the second direction a solution of ammonium phosphate of pH 6.5, $[NH_4^+] = 0.4$ (System II), was used. The times of continuous ascending development[6] were 3 h with System I and 2.5 h with System II.

The spots were rendered visible under a short-wave (254 nm) UV lamp (Desaga Uvis).

RESULTS AND DISCUSSION

The fundamental point of separation involving the hydrogen and the salt forms of a given cation-exchange thin layer in two-dimensional combination is the exchange

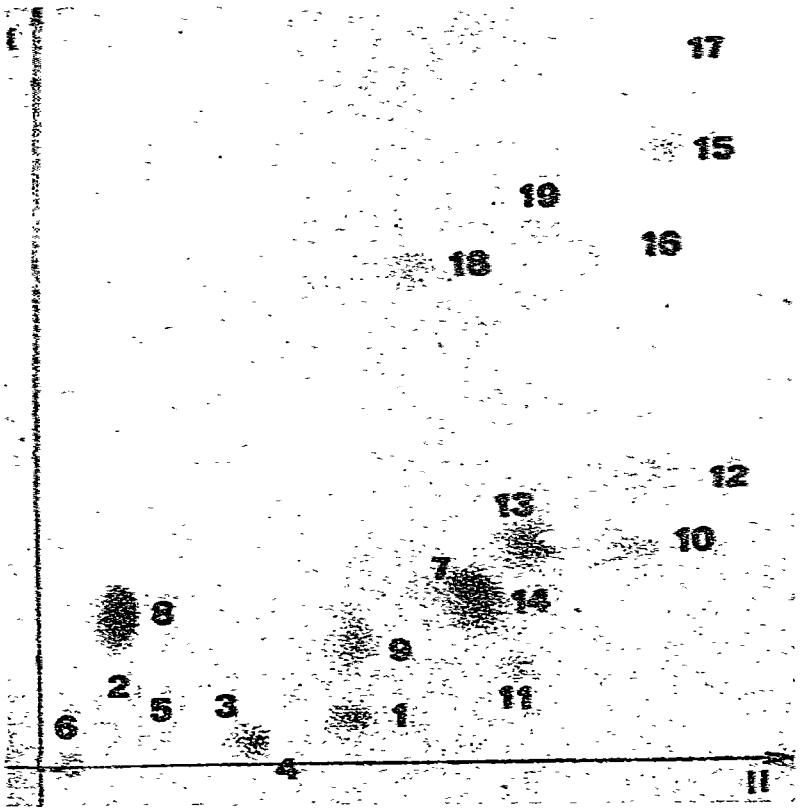

Fig. 1. Two-dimensional separation of purine and pyrimidine bases on strong cation-exchange thin layers; for details see text. 1 = Adenine; 2 = 1-methyladenine; 3 = 2-methyladenine; 4 = N^6-methyladenine; 5 = N^6-dimethyladenine; 6 = N^6-(isopent-2-enyl)adenine; 7 = cytosine; 8 = 3-methylcytosine; 9 = 5-methylcytosine; 10 = 5-(hydroxymethyl)cytosine; 11 = 2-thiocytosine; 12 = xanthine; 13 = hypoxanthine; 14 = 1-methylhypoxanthine; 15 = uracil; 16 = 3-methyluracil; 17 = 5-(hydroxymethyl)uracil; 18 = 5-bromouracil; 19 = thymine.

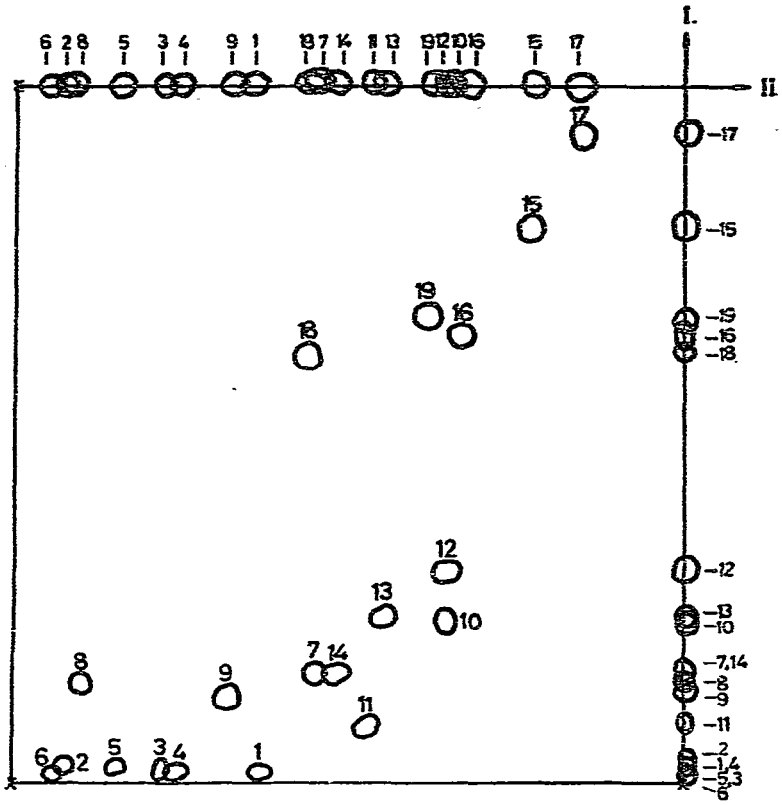

Fig. 2. Behaviour of purine and pyrimidine bases in cation-exchange TLC systems I and II, and two-dimensional chromatogram drawn on the basis of one-dimensional separations. For numbering of spots see Fig. 1; for details see text.

of counter-ions between the two developments in a way such as to retain the pattern of separation established during the first development. Obviously, successful implementation is attained when a layer in the hydrogen form is used for the first dimension and transformation of the exchanger into the salt form is performed in the "vapour phase", *i.e.*, by the action of the vapour of a volatile base. Consequently, only cations derived from volatile bases can be applied as counter-ions for the second development; ammonium ions were chosen for this purpose to ensure simplicity of operation.

It was found that about 85% of the hydrogen counter-ions were exchanged for ammonium ions, when a strong cation-exchange layer was placed for 10 min at room temperature in a closed vessel saturated with ammonia vapour. The degree of conversion could not be increased by prolonging the time of treatment; this, however, was unimportant, as use of the partially converted layer gave adequate results.

The resolution of nine purine and ten pyrimidine bases, obtained by combining TLC systems I and II, is shown in Fig. 1. For comparison, a two-dimensional chromatogram drawn on the basis of one-dimensional separations in the individual systems is shown in Fig. 2. It can be seen from Fig. 2, that neither system was capable of separating the bases in one dimension; system II gave much better results than

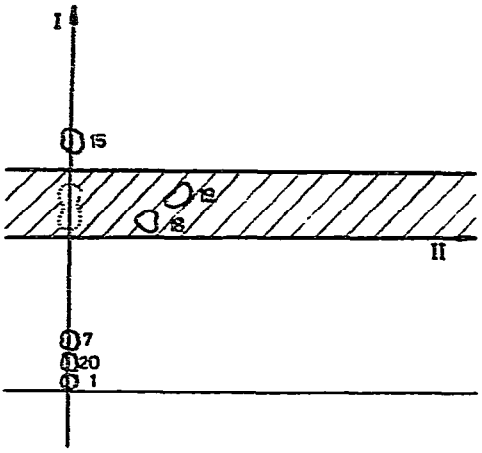

Fig. 3. Two-dimensional separation of a mixture of the four DNA bases, uracil and 5-bromouracil on strong cation-exchange thin layers. For details see text. 1 = Adenine; 7 = cytosine; 15 = uracil; 18 = 5-bromouracil; 19 = thymine; 20 = guanine.

system I, but, even with system II, resolution between sets of structurally similar compounds was only partial, and only a few were obtained as distinct homogeneous spots. On the other hand, almost complete resolution of all nineteen bases was attained by combining the two systems (see Fig. 1). The pattern of separation was similar to, but better than, that expected on the basis of Fig. 2, unsatisfactory resolution occurring only between the spots of 2-methyladenine and N^6-methyladenine, although there was some overlap between cytosine and 1-methylhypoxanthine. It should be noted, however, that bases present in the layer were unaffected by the counter-ion conversion procedure. Thirteen of the nineteen bases studied have been separated by two-dimensional partition TLC on silica gel and Avicel microcrystalline cellulose[12]; in these instances, the spots for adenine and 1-methylhypoxanthine overlapped.

Another example of the application of the technique is the resolution of a mixture of the four DNA bases and 5-bromouracil. It is well known that 5-bromo-2′-deoxyuridine (the structural analogue of deoxythymidine) can be incorporated in DNA of different organisms[13]. In the base analysis of such modified DNA, separation of the above-mentioned five compounds has usually been achieved by two-dimensional partition TLC on cellulose[14]; here, an alternative method is offered. As shown in Fig. 3, a mixture of the four DNA bases, 5-bromouracil and uracil could be satisfactorily resolved by using systems I and II. In system I, only thymine and 5-bromouracil remained unresolved (dotted area in Fig. 3), so that only the narrow zone of the chromatoplate containing this mixed spot was needed for the second development (shaded area in Fig. 3). It should be noted, however, that the whole area could not be used, owing to the "heavy" tailing of guanine in system II.

ACKNOWLEDGEMENT

The invaluable technical assistance of Miss G. Gémes is gratefully acknowledged.

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