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The Qualitative Separation of Purines, Pyrimidines, and Nucleosides by Paper Chromatography

NILS LÖFGREN

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Stockholm, Sweden

In recent yers much work has been done on paper chromatographic analysis of "nucleo" derivatives [1–7]. The separation into individual components of the mixtures of purines, pyrimidines, and nucleosides as they commonly occur in nature seems, however, to have received but little attention. Hotchkiss' work showed that water-saturated butanol can resolve certain combinations into their components. In connection with our attempts to isolate nebularine — an antibiotic active against *Mycobacteria* — it became necessary to carry out qualitative analyses of complicated systems containing purines, pyrimidines, and nucleosides. We therefore decided to try to separate qualitatively by means of paper chromatography, the 12 common derivatives, *i.e.* adenine, adenosine, guanine, guanosine, xanthine, hypoxanthine, uracil, uridine, cytosine, cytidine, thymine, and thymidine. We believe that almost any combination of these substances may be resolved into its components by our method.

EXPERIMENTAL

Since some purine and pyrimidine derivatives are weakly acid and others weakly basic, we chose solvent mixtures possessing both acid and basic characteristics. The spots on the paper were located by means of Holiday and Johnson's ultraviolet method [8] (*cf.* below). In addition to possessing good resolving power, the liquids must not absorb radiation in the wave-length region in question *. After many experiments we were able to select four different liquids which together fulfil the necessary conditions. We call them here the isobutyric acid, butanol-isobutyric acid, morpholine, and piperidine liquids.

* It might appear *a priori* possible to remove the liquid from the paper strip (by drying, washing) sufficiently for any possible absorption not to interfere with the analysis. Judging from our experiments, however, this does not seem to be easy in practice.

The compositions of the four mixtures are given below; the time in hours required for the liquid front to reach a distance of 49—50 cm from the starting line is also stated (h/49—50 cm). These figures refer to Whatman No. 4 filter paper strips and to a temperature of 22°C.

The isobut. acid liquid:

Isobutyric acid	400 ml
Water	208 ml
Ammonia, 25 %	0.4 ml

h/49—50 cm = 12 ± 2

The butanol-isobut. acid liquid:

n-Butanol	75 ml
Isobutyric acid	37.5 ml
Water	25 ml
Ammonia, 25 %	2.5 ml

h/49—50 cm = 13 ± 2

The morpholine liquid:

n-Butanol	90 ml
Morpholine	30 ml
Diethylene glycol	20 ml
Water	40 ml

h/49—50 cm = 22 ± 3

The piperidine liquid:

n-Butanol	80 ml
Piperidine	30 ml
Diethylene glycol	10 ml
Diethyl carbitol	20 ml
Water	40 ml

h/49—50 cm = 16 ± 2

The isobutyric acid liquid was used by Stjernholm [9] for paper chromatographic analysis of various "nucleo" derivatives. We used the morpholine liquid of Vischer and Chargaff [3], who used it to separate certain of the purine and pyrimidine bases (no nucleosides being reported). The commercial liquid components were purified by distillation, a fraction corresponding to a boiling-point interval of 2° being used as a rule; the diethylene glycol was distilled within the interval 130—134° at 14 mm Hg. In preparing the liquids, the individual components should be measured out very carefully. The butanol-isobutyric acid liquid undergoes certain changes with time, but if the mixture is kept in a refrigerator, no variation in the R_F values can be observed even after two months.

The method of descending paper strip chromatography was followed, a standardized procedure being applied. The paper used was Whatman No. 4 (available in 57 × 46.5 cm). The filter paper was cut into strips 57 cm long and 7 or 9 cm wide depending on the trough used. Two transverse lines were drawn with a pencil 50 and 60 mm, respectively, from one end of the strip. The line at 60 mm is the starting line, along which faint points were marked with a pencil, never closer than 2 cm to the edge of the strip (usually 2.3 cm). The solutions to be investigated were then deposited as spots on these points. On the narrower strips two or three such points were marked, on the broader ones three or four points.

The experiments were carried out in glass chambers placed in a room that could be kept at 22.0 ± 0.5° *. Each chamber consisted of a glass cylinder with a plane upper surface covered with a glass disk tightly joined to the edge of the cylinder by means of silicone grease. A little hole was bored in the glass disk and plugged with a cork. The glass trough could be filled through a funnel inserted in the hole. The trough was supported by a scaffolding of pyrex glass rods standing on the bottom of the chamber. The

* If the temperature varies more than that, the R_F values obtained will show larger errors than those stated here.

Table 1. R_F values at 22° C for purines, pyrimidines and nucleosides on Whatman No. 4 measurements for each

Solvent mixture	Adenine	Adenosine	Guanine	Guanosine	Xanthine
Butanol-isobut. acid liquid	0.56 ± 0.01 (4)	0.72 ± 0.02 (5)	0.39 ± 0.01 (4)	0.27 ± 0.02 (4)	0.34 ± 0.01 (4)
Isobut. acid liquid	0.83 ± 0.015 (4)	0.91 ± 0.02 (5)	0.70 ± 0.02 (5)	0.59 ± 0.03 (5)	0.60 ± 0.02 (5)
Morpholine liquid	0.64 ± 0.02 (3)	0.57 ± 0.01 (4)	0.36 ± 0.02 (4)	0.42 ± 0.01 (4)	0.34 ± 0.01 (4)
Piperidine liquid	0.56 ± 0.025 (5)	0.56 ± 0.03 (5)	0.23 ± 0.03 (5)	0.25 ± 0.03 (5)	0.24 ± 0.015 (4)

bottom was covered with the solvent mixture a sufficient length of time ahead of the test for the equilibrium liquid—vapour to be established. The bottom liquid may be used many times, provided care is taken not to keep the chamber unnecessarily exposed to the air.

The solutions to be investigated usually contained 5 mg/4 ml of each individual compound. These solutions were prepared by heating the substance in water at 100°, adding if necessary concentrated ammonia or dilute hydrochloric acid drop by drop from a micropipette *.

The R_F values of the individual compounds remain unchanged whether ammonia or hydrochloric acid is used, provided the routine treatment of the spots with ammonia gas is adhered to (*cf.* below).

* Of the twelve substances, only guanine requires the addition of hydrochloric acid; the others are easily dissolved in water or weak ammonia solution.

filter paper: mean values with maximal deviation; figures in brackets denote the number of individual compound.

Hypo-xanthine	Cytosine	Cytidine	Uracil	Uridine	Thymine	Thymidine
0.45 ± 0.01 (5)	0.60 ± 0.01 (4)	0.39 ± 0.015 (10)	0.50 ± 0.01 (3)	0.34 ± 0.025 (11)	0.63 ± 0.02 (6)	0.60 ± 0.01 (6)
0.69 ± 0.02 (5)	0.80 ± 0.02 (5)	0.73 ± 0.015 (10)	0.67 ± 0.02 (6)	0.60 ± 0.02 (11)	0.78 ± 0.01 (6)	0.75 ± 0.02 (6)
0.46 ± 0.02 (3)	0.57 ± 0.01 (3)	0.61 ± 0.02 (9)	0.62 ± 0.02 (3)	0.65 ± 0.03 (10)	0.81 ± 0.02 (6)	0.86 ± 0.01 (6)
0.37 ± 0.03 (5)	0.42 ± 0.01 (5)	0.44 ± 0.02 (5)	0.44 ± 0.03 (5)	0.35 ± 0.02 (5)	0.54 ± 0.03 (5)	0.54 ± 0.025 (5)

The solutions were applied with micropipettes to the filter strips at the points marked. The spots were allowed to spread to a diameter of about 7—9 mm, corresponding to a liquid volume of about 2—3 μl, *i. e.* each spot contained about 2.5—4 γ of each individual compound. The spots were then air-dried for 15 minutes. Ammonia gas was blown through a capillary tube at every spot for 15 seconds from either side. The paper was then allowed to stand for 20 minutes in air, before inserting it in the chamber. A glass rod about 6 mm in diameter was placed between the two pencilled lines, one of which is the starting line (*cf.* above), and the strip then folded over it. The strip was then placed in the *empty* trough in such a way that the line above the starting line lay over the inner edge of the trough. After the strips were inserted, the chamber was closed. One hour was allowed for equilibration of the paper with the liquid-vapour athmosphere, after which the solvent mixture was added to the trough through the funnel inserted in the hole in the glass disk. When the liquid front had reached about 1 cm from the lower edge of the strip (about 50 cm from the starting line), the experiment was interrupted. The strips

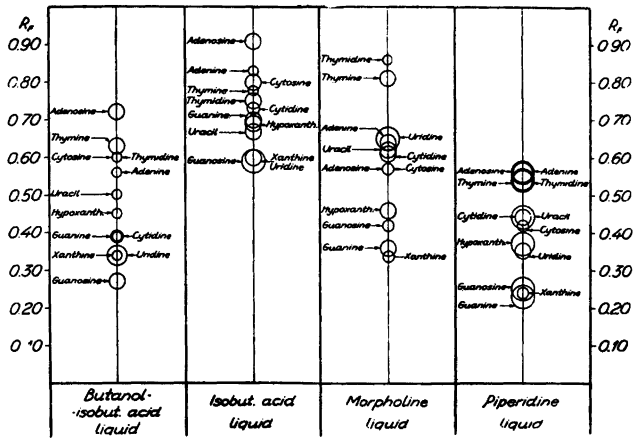

Fig. 1. Schematic presentation of R_F values at 22° C for purines, pyrimidines and nucleosides on Whatman No. 4 filter paper; each compound symbolized by a circle, the radius of which indicates maximal deviation from the mean value.

were taken out and the liquid front marked *. The strips were then dried in a suitable cabinet through which hot air from an ordinary hair dryer was blown for 25—30 minutes.

A masonite box 14 cm long and wide, and 60 cm high was used for the purpose. The strips were hung in this cabinet and hot air from an ordinary hair dryer, inserted through the lid, was blown in from above.

The dried strips were examined by Holiday and Johnson's method [8], *i. e.* the strips were exposed to a suitable ultraviolet lamp, the spots appearing as dark patches against the background of paper fluorescence ** .The boundaries of the dark patches were marked with a pencil. When applying 2.5—4 γ the dark patches are as a rule seen very distinctly. When using morpholine liquid (in which case the paper fluoresces rather strongly) some of the compounds are seen less distinctly. Therefore, it may be advisable when using this liquid for an untrained observer to double the quantity of substance in the spots; this change in the content of the spots does not change the R_F values.

RESULTS

The R_F value of each individual compound was determined by three or more measurements. The results are shown in Table 1, which gives the mean R_F values, the maximal deviation from the mean, and the number

* The solvent mixture in the trough may without detriment be returned to the original solution, care only being taken that the content of the trough is exposed to air for as short a time as possible. As previously mentioned, the bottom liquid is left in the *carefully closed* chamber; it may be used for a large number of subsequent tests.

** It was found that the argon-mercury lamp made by AB Luma Lamp, which gives a UV-radiation of almost exclusively 254 mμ, was excellent for the purpose.

of measurements. Fig. 1 gives a schematic diagram of the results; each substance is represented by a circle, the radius of which is an indication of the maximal deviation from the mean value.

When carrying out analyses of mixtures containing various mixtures of the twelve purine and pyrimidine derivatives, the R_F value of each individual compound does not change. Further, it is evident from these experiments that the components of almost any mixture of the twelve compounds can be separated and identified.

SUMMARY

By paper strip chromatography it has been possible to resolve and identify the components of almost any composited mixture of twelve natural purine and pyrimidine derivatives, *viz.* adenine, adenosine, guanine, guanosine, xanthine, hypoxanthine, cytosine, cytidine, uracil, uridine, thymine, and thymidine.

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