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The Separation of N-2:4-Dinitrophenyl Amino-Acids on Paper Chromatograms

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The method of identification and estimation of the free amino-groups of proteins, first applied to insulin by Sanger (1945), has since been widely used. The terminal amino-acids of the peptide chains which carry free amino groups are isolated as their N-2:4-dinitrophenyl (DNP) derivatives, which can be separated on silica gel partition chromatograms (Sanger, 1945). Buffered columns may also be employed with a variety of solvent systems (Blackburn, 1949).

A number of attempts to separate DNP-amino-acids on paper chromatograms have met with only limited success due to 'tailing' of the spots (Sanger, 1945; Blackburn, 1949). Phillips & Stephen (1948) separated some of the DNP-amino-acids, particularly the slower moving ones, using a two-dimensional method, but also observed marked 'tailing' in many solvents. In contrast they found that fairly large DNP peptides formed good spots on paper chromatograms. Woolley (1949) also found that some DNP peptides from enzymic digests of DNP-insulin ran as single spots on phenol-ammonia or butanol-ammonia chromatograms.

We have now observed that DNP-amino-acids may be successfully separated on buffered one-dimensional paper chromatograms, compact spots without 'tailing' and possessing characteristic rates (R_F values) being formed in suitable solvents. After the present paper had been read to the Biochemical Society (Blackburn & Lowther, 1950) we received from Prof. C. Fromageot a copy of a paper by Monier & Pénasse (1950), who separated mixtures of DNP-amino-acids on paper using solvents saturated with potassium benzoate solution.

EXPERIMENTAL

The chromatograms were run on strips, approx. 12 × 35 cm., of Whatman no. 4 filter paper which had been previously soaked in buffer solution and dried at room temperature.

Phthalate buffer of pH 6.0 (Britton, 1942, Table 70) proved generally suitable, and was used except where otherwise stated. About 2–5 µg. of a DNP-amino-acid (dissolved in a suitable solvent) was applied to the paper. The papers were then fixed in small stainless steel troughs mounted in glass battery jars, water and solvent being placed in the bottom of the jar, and the top covered with a sheet of plate glass. The closed box was allowed to stand for some time before the solvent (previously saturated at room temperature with the same buffer that was applied to the paper) was introduced into the trough. The movement of the DNP-amino-acids down the paper could be observed during the course of the experiment. Adequate separations were generally obtained in a run lasting several hours, or occasionally overnight.

Solvent systems

Many of the solvent systems tried proved unsuitable, because the DNP-amino-acids ran fast near the solvent front or else 'tailed' somewhat. The solvent systems of general use are described below.

tert.-Amyl alcohol. All the DNP-amino-acids move as well defined spots in this solvent.

Propanol-cyclohexane. A mixture of DNP-leucine, DNP-valine, DNP-alanine and DNP-glycine separates into four clearly defined spots in 30% propanol-cyclohexane (Fig. 1). Occasionally difficulty may be experienced with this solvent, owing to the volatility of cyclohexane, and 'tailing' spots result. This may be overcome by substituting light petroleum (b.p. 100–120°) for the cyclohexane, when similar separations can be achieved.

n-Butanol and benzyl alcohol. The spots formed in these solvents are slightly elongated, but the addition of 10% of ethanol (v/v) before saturation with buffer causes more compact spots to be formed. 10% ethanol-benzyl alcohol is superior to 10% ethanol-butanol, as the differences in R_F values between the different DNP-amino-acids are greater.

Ethyl acetate and 1% butanol-chloroform are not as generally useful as the above solvents. Ethyl acetate may be used for the separation of the slower moving DNP-amino-acids on pH 8 phosphate-citrate buffer paper (Britton, 1942, Table 65). DNP-threonine and DNP-serine can be separated from each other and from DNP-glutamic acid and DNP-aspartic acid, both of which move slowly. The DNP-mono-

amino-acids, however, give rather elongated spots in this solvent. DNP-threonine, DNP-serine, DNP-glutamic acid and DNP-aspartic acid do not move in 1% butanol-CHCl₃, while the DNP-monoamino-acids move fairly fast down the paper. DNP-glycine and DNP-alanine, however, may be separated from each other.

the same reason, but Table 1 gives some values for a few representative solvents.

It should be possible to perform end-group estimations on a micro scale by comparing the size and intensity of the DNP-amino-acid spots with those given by known amounts of DNP-amino-acids run simultaneously on the same chromatogram.

Fig. 1. Chromatogram of DNP-amino-acids run in 30% propanol-cyclohexane.

The R_F values of the individual DNP-amino-acids vary with the pH of the buffer applied to the paper, but slight variations do not prevent the necessary separations. The R_F values also appear to vary with the temperature, probably due to alteration in the composition of the solvent mixtures.

Table 1. The R_F values of DNP-amino-acids on buffered paper

	Solvent system		
	30% Propanol- cyclohexane	Tertiary amyl alcohol	10% Ethanol- benzyl alcohol
DNP-leucine	0.28	0.88	0.71
DNP-valine	0.23	0.79	0.59
DNP-phenylalanine	0.22	0.74	0.63
DNP-alanine	0.15	0.46	0.36
DNP-glycine	0.10	0.23	0.26
DNP-threonine	0.07	0.36	0.26
DNP-serine	0.05	0.21	0.18
DNP-glutamic acid	0.05	0.04	0.07
DNP-aspartic acid	0.02	Slow	0.03

For these reasons, identification of a DNP-amino-acid is never based on the R_F values alone, control DNP-amino-acids always being run on the same sheet of paper as the unknown. Extensive Tables of R_F values are not quoted for

gram. After drying, photographic copies of the chromatograms may be made on contact paper, when the spots show up clearly. A hydrolysate of DNP-insulin, when examined in 30% propanol-cyclohexane, gave two spots in a similar position to those given by a mixture of DNP-phenylalanine and DNP-glycine. Phenylalanine and glycine are known to be the terminal amino-acid residues of insulin (Sanger, 1945).

DISCUSSION

The DNP-amino-acids run most successfully on paper in solvents where the mobile phase has a high water content. This high water content may prevent association of the DNP-amino-acids, which can lead to 'tailing'. The phthalic acid of the buffer, which is slightly soluble in the mobile phase, probably also helps in preventing this association (cf. Baker, Dobson & Martin, 1948). When the amount of water in the mobile phase of a two-component system such as benzyl alcohol-water is increased by the addition of a third component, such as ethanol which is soluble in both phases, the DNP-amino-acid spots become much more compact in shape. If the water content of the mobile phase is too high, the R_F values are large and the difference between the values for different DNP-amino-acids is not sufficient to effect

separations. This occurs with *sec.*-butanol saturated with pH 6 buffer.

When DNP-amino-acids are run on paper with water or buffer as solvent, they travel down the paper as 'spots' some distance behind the solvent front. With methanol or ethanol on pH 6 buffered paper, fairly well-defined 'spots' with some 'tailing' are formed, the separation between individual DNP-amino-acids being greater than in water alone. When non-hydroxylic organic solvents not containing water are similarly used, 'tailing' spots are formed.

SUMMARY

A method for the separation of *N*-2:4-dinitrophenyl amino-acids on buffered paper chromatograms is described.

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