# Water-miscible Solvents in the Separation of Amino-acids by Paper Chromatography

By H. R. BENTLEY and J. K. WHITEHEAD
*Research Association of British Flour-Millers, Cereals Research Station, St Albans*

(*Received* 29 *September* 1949)

The separation of amino-acid mixtures into discrete spots on paper chromatograms has hitherto almost invariably been carried out using solvents only partially miscible with water. Our work has entailed the frequent use of *s*-collidine as a developing solvent. This solvent is objectionable chiefly on account of its offensive smell, because of possible toxic effects (Ludwig, 1935; Holtzmann, 1936) and also because it frequently gives 'ghost' spots and double spots which are confusing when dealing with unknown products of protein hydrolysis. In an effort to replace collidine we have examined many other solvents both partially miscible and totally miscible with water. Edman (1946) found that a mixture of equal volumes of pyridine and amyl alcohol had practically the same resolving capacity as collidine and could with advantage replace this solvent. Consden, Gordon & Martin (1944) stated originally that solvents completely miscible with water can be used provided the water content of the solvent is not too high, but that even then the amino-acid bands are too broad to be of value. We have confirmed that this is so, but only when volatile solvents are used to develop the chromatogram by downward irrigation from horizontal troughs in the conventional apparatus (Consden *et al.* 1944; Dent, 1948). In this type of apparatus, with large air space, it is inevitably some time after the fitting of the canopy or the lid before the atmosphere becomes saturated with solvent and water vapour. During this time preferential evaporation of a volatile solvent occurs from the surface of the paper as the water-saturated solvent travels along it. This leaves the paper waterlogged. Goodall & Levi (1947) overcame this difficulty with wet ether in the separation of penicillins by devising an airtight container with a much reduced air space and equilibrating the contents of the apparatus at 0°. Karnovsky & Johnson (1949), for the same purpose, used an airtight container in which equilibration was completed before addition of solvent. This was done through an inlet tube without having to open the main container. Non-volatile water-miscible solvents can, however, be used in the conventional manner, and in a preliminary communication (Bentley & Whitehead, 1949) we have described the successful use of two such solvents. This work has now been extended to include several other solvents which are comparatively volatile and which are satisfactory if run by ascending capillary irrigation in sealed glass tanks (Williams & Kirby, 1948).

Arden, Burstall, Davies, Lewis & Linstead (1948) have used water-miscible solvents for the separation of inorganic ions, and recently Hanes & Isherwood (1949) have used them for the separation of phosphoric esters. On starch columns, Moore & Stein (1949) have separated amino-acids using water-miscible solvents.

The water-miscible solvents employed successfully in the present work include methanol, ethanol, *n*-propanol, acetone, pyridine, tetrahydrofuran, furfuryl alcohol and tetrahydrofurfuryl alcohol. Dioxan failed to give satisfactory spots; veratryl alcohol is insufficiently volatile to be dried easily; allyl alcohol is satisfactory but extremely unpleasant for routine use.

## MATERIALS AND METHODS

*Solvents.* Acetone and pyridine are of A.R. quality. Methanol, ethanol and *n*-propanol are the commercial absolute alcohols. Furfuryl alcohol is freshly distilled *in vacuo* before use, since the clarity of the final chromatographic picture is seriously impaired if the solvent has acquired a yellow coloration; the inclusion of urea (0·5%, w/v) as a stabilizer (Wilson, 1947) sharpens the outlines of the spots and reduces their size. Commercial tetrahydrofurfuryl alcohol and tetrahydrofuran are used without further purification. Phenol used with these solvents in two-way chromatograms is prepared by saturating Liquified Phenol (B.P.) with water.

*Apparatus.* With furfuryl alcohol and tetrahydrofurfuryl alcohol conventional apparatus is employed. The solvent flows downward from a horizontal trough over a sheet of filter paper (18½ × 22 in.) suspended vertically and enclosed in a canopy (30 × 30 × 5 in.) (cf. Consden *et al.* 1944; Dent, 1948). With volatile solvents the method of Williams & Kirby (1948) is used. Amino-acid spots are placed 6 cm. from the shorter edge of a sheet of filter paper (18½ × 20 in.) and at least 10 cm. from the longer edges where solvent flow is uneven. The longer edges of the sheet are joined with wire staples to form a hollow cylinder. This is placed vertically in a glass tank (12 × 8 × 21 in.) the floor of which is covered to a depth of approximately 0·5 in. with solvent (about 600 ml.) which can be re-used for several successive runs, but which must be frequently replaced because of changes in composition due to unequal evaporation of water and solvent. The open end of the tank is carefully sealed by means of a

greased glass plate to keep the internal atmosphere saturated with solvent and water vapour. The tanks are kept in a constant-temperature room at 18·3 ± 0·5°.

*Paper.* Whatman no. 1 paper is used in the glass tanks, when the solvent front reaches a convenient height (30–35 cm.) in about 15 hr. (overnight); furfuryl alcohol and tetrahydrofurfuryl alcohol move at approximately the same rate by downward flow. On Whatman no. 4 paper the solvents move more quickly, reaching a similar point after about 8 hr.

*Visualization of spots.* The positions of the amino-acid spots are revealed by spraying the dried papers with a solution of ninhydrin (0·1%, w/v) in dry *n*-butanol in the usual manner. In the case of furfuryl alcohol, if the paper is dried at too high a temperature, particularly in the presence of furoic acid, brown stains appear in the area of the spots which then fail to develop the characteristic colours; further, with this solvent rapid fading of the colours occurs and the spots should therefore be marked immediately after development.

## RESULTS

Under the conditions described above most of the amino-acids give small, sharply defined spots. With the basic amino-acids, applied as hydrochlorides, the spots are sometimes elongated and lysine and ornithine often give long streaks; where this occurs no $R_F$ value (Consden *et al.* 1944) is included for the amino-acid in Table 1 and Figs. 1 and 2. Edman (1946) and Dent (1948) have already noted this effect in the case of basic amino-acids. Cystine often gives an unsatisfactory spot and tyrosine streaks with furfuryl alcohol-pyridine. The addition of urea (0·5%, w/v) to acetone and furfuryl alcohol improves the definition of the spots, but has little effect on $R_F$ values.

The $R_F$ values for the amino-acids with the various solvents, together with the amounts of water (v/v) and other additions named are given in Table 1. As pointed out by Dent (1948), the values will vary (up to 15%) with the conditions of a particular experiment and according to the type of apparatus used. The relative positions of the spots, however, remain the same, and for each experiment known amino-acids should be used as markers under conditions identical with those used for unknown substances. $R_F$ values obtained with Whatman no. 1 and Whatman no. 4 papers are approximately the same. It will be seen that almost identical results are given by acetone and pyridine; for routine use the former is obviously to be preferred. Additions of pyridine and of furoic acid to furfuryl alcohol give useful variations in $R_F$ values. The addition to acetone of hydrochloric, acetic, trichloroacetic or benzenesulphonic acids either causes excessive streaking or else the amino-acid spots all progress with the solvent boundary.

The effect of increasing water content on the $R_F$ values in furfuryl alcohol is illustrated in Fig. 1. The actual magnitudes of the $R_F$ values, but not the sequence, vary with the amount of water present, increasing with increasing water content whilst the range of values decreases. We have used furfuryl alcohol containing up to 80% water (v/v). At this high level there is little if any separation of the amino-acids and the bands are very broad, but at 60% water content there is some differentiation of $R_F$ values and the spots are reasonably compact. With all solvents streaking occurs to a greater or lesser extent below 20% (v/v) water content and the optimum water content for small, sharply defined spots lies within the range 20–40% (v/v).

Fig. 2, in which the amino-acids are arranged in ascending order of $R_F$ value in water-saturated *n*-butanol, gives a comparison between this solvent and the three lower water-miscible alcohols whose aqueous solutions we have studied. Whilst the general pattern is broadly similar in each case, the amino-acids are seen to move more slowly with increasing molecular weight of the alcohols and the variations in $R_F$ value for different amino-acids become less.

Each of the water-miscible solvents we have used behaves normally in two-way paper chromatograms. Acetone containing water (40%, v/v) has proved particularly useful in our work, replacing collidine as a second solvent for chromatograms run first with phenol. In work connected with the isolation of a toxic factor from $NCl_3$-treated zein (Bentley, Macdermott, Pace, Whitehead & Moran, 1949) a fraction was obtained giving a single spot on paper chromatograms with *tert*-amyl alcohol, *n*-butanol, *n*-butanol-acetic acid, benzyl alcohol, furfuryl alcohol or phenol. With aqueous acetone three distinct spots with well-spaced $R_F$ values were obtained. Collidine gave a similar pattern, but the occurrence of double spots and haloes with this solvent, as well as the very low $R_F$ values, made interpretation ambiguous. Further purification yielded crystalline material giving a single spot in aqueous acetone, thus affording some evidence for the homogeneity of the material.

## DISCUSSION

The fact that typical separations can be effected on paper chromatograms with water-miscible solvents as well as with solvents only partially miscible with water raises the question of the mechanism of paper chromatography as applied to amino-acids. Consden *et al.* (1944) originally postulated a partition mechanism for the paper chromatogram, whereby the solute occupied a position on the chromatogram determined by its partition coefficient between the flowing solvent and the water phase supported in the cellulose fibres. These authors suggested that, in the case of water-miscible solvents containing small amounts of water, a 'salting-out' effect occurred, allowing the system to function as a partition chromatogram. Arden *et al.* (1948) suggested that this explanation was too simple and that other factors were operative, such as selective extraction of the mixture by the solvent at the site of the test spot and adsorption of the material by the paper. Our results agree particularly well with a broader concept of the mechanism of paper chromatography recently developed by Hanes & Isherwood (1949). These authors stress the fact that the water held in filter paper cannot be considered as free liquid water, but is bound in some way, possibly by hydrogen bonding, to the hydrophilic hydroxyl groups of the cellulose. They suggest that the solute molecules, by virtue of their hydrophilic groups, will compete with water and solvent molecules, which usually contain hydrophilic groups also, in the mobile phase for incorporation in this cellulose-water complex. The distribution of any given solute (and hence the $R_F$ value) will depend upon such factors as the number,

Table 1. *$R_F$ values of amino-acids with water-miscible solvents*

(Colours given by ninhydrin, blue (*B*); brown (*Br*); grey (*G*); purple (*P*); yellow (*Y*). The colours given in all the solvents except furfuryl alcohol are the same as with pyridine.)

| Solvent | Tetrahydrofuran | Tetrahydrofurfuryl alcohol | Pyridine | Acetone | Acetone | Furfuryl alcohol | Furfuryl alcohol | Furfuryl alcohol | Furfuryl alcohol |
|---|---|---|---|---|---|---|---|---|---|
| Water and other additions (v/v) | 40% water | 20% water | 35% water | 40% water | 40% water + 0·5% w/v) urea | 25% water | 25% water + 0·5% (w/v) urea | 25% water + 8% pyridine | 25% water + 5% furoic acid |
| Paper, Whatman no. | 1 | 4 | 1 | 1 | 1 | 4 | 4 | 4 | 4 |
| Direction of running | Ascending | Descending | Ascending | Ascending | Ascending | Descending | Descending | Descending | Descending |
| Aspartic acid | 0·38 | 0·15 | 0·43 *B* | 0·45 | 0·38 | 0·18 | 0·20 *B* | 0·07 | 0·23 |
| Glutamic acid | 0·45 | 0·19 | 0·48 *P* | 0·51 | 0·46 | 0·18 | 0·23 *P* | 0·09 | 0·33 |
| Cystine | — | 0·20 | — | — | — | 0·16 | 0·19 *G* | 0·06 | 0·11 |
| Serine | 0·48 | 0·34 | 0·51 *P* | 0·47 | 0·44 | 0·20 | 0·29 *P* | 0·14 | 0·26 |
| Glycine | 0·44 | 0·28 | 0·41 *RP* | 0·45 | 0·42 | 0·21 | 0·28 *Br* | 0·15 | 0·29 |
| Ornithine* | 0·30 | 0·31 | 0·29 *P* | 0·16 | 0·19 | 0·24 | 0·28 *P* | 0·10 | 0·24 |
| Arginine* | 0·34 | 0·27 | 0·31 *P* | 0·15 | 0·20 | 0·34 | 0·32 *P* | 0·17 | 0·32 |
| Histidine* | 0·59 | 0·31 | 0·43 *GP* | 0·38 | 0·33 | 0·25 | 0·39 *Br* | 0·22 | 0·31 |
| Lysine* | — | — | — | — | — | 0·25 | 0·20 *P* | 0·13 | 0·28 |
| Alanine | 0·67 | 0·43 | 0·54 *P* | 0·55 | 0·52 | 0·34 | 0·39 *PB* | 0·26 | 0·47 |
| Threonine | 0·66 | 0·46 | 0·56 *P* | 0·57 | 0·50 | 0·35 | 0·34 *P* | 0·22 | 0·37 |
| Hydroxyproline | 0·63 | 0·39 | 0·56 *Y* | 0·53 | 0·54 | 0·35 | 0·41 *Y* | 0·32 | 0·43 |
| Tyrosine* | 0·72 | 0·59 | 0·65 *GP* | 0·63 | 0·69 | 0·46 | 0·47 *G* | — | 0·52 |
| Valine | 0·73 | 0·64 | 0·65 *P* | 0·62 | 0·65 | 0·50 | 0·52 *BP* | 0·43 | 0·64 |
| Methionine | 0·66 | 0·67 | 0·66 *P* | 0·63 | 0·65 | 0·55 | 0·58 *P* | 0·42 | 0·59 |
| Proline | 0·73 | 0·49 | 0·56 *Y* | 0·58 | 0·62 | 0·56 | 0·56 *Y* | 0·42 | 0·61 |
| Tryptophan* | 0·78 | 0·70 | 0·63 *Br* | 0·58 | 0·64 | 0·56 | 0·59 *GP* | 0·43 | 0·61 |
| Leucine | 0·78 | 0·72 | 0·68 *P* | 0·69 | 0·71 | 0·59 | 0·63 *P* | 0·43 | 0·69 |
| Isoleucine | 0·78 | 0·71 | 0·66 *P* | 0·67 | 0·71 | 0·59 | 0·63 *P* | 0·43 | 0·70 |
| Phenylalanine | 0·79 | 0·73 | 0·66 *P* | 0·64 | 0·67 | 0·63 | 0·64 *GP* | 0·52 | 0·72 |

* These amino-acids were put on the papers as hydrochlorides.

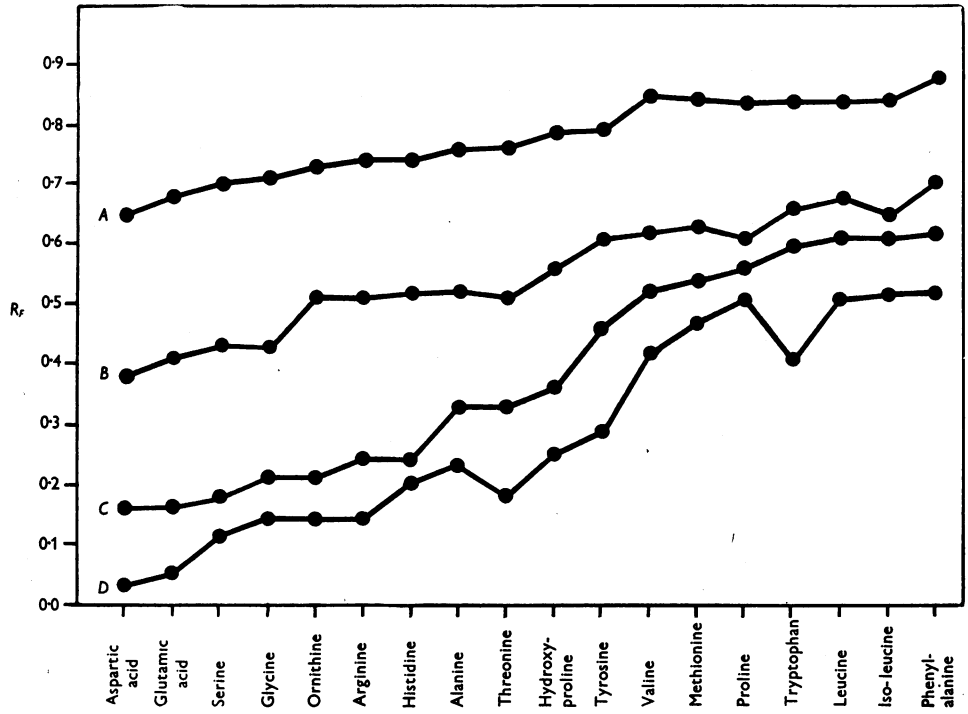


Fig. 1. Effect of water content of furfuryl alcohol on the $R_F$ values of amino-acids on Whatman no. 4 paper at room temperature. (Water content (v/v): *A*, 60%; *B*, 40%; *C*, 30%; *D*, 10%.)

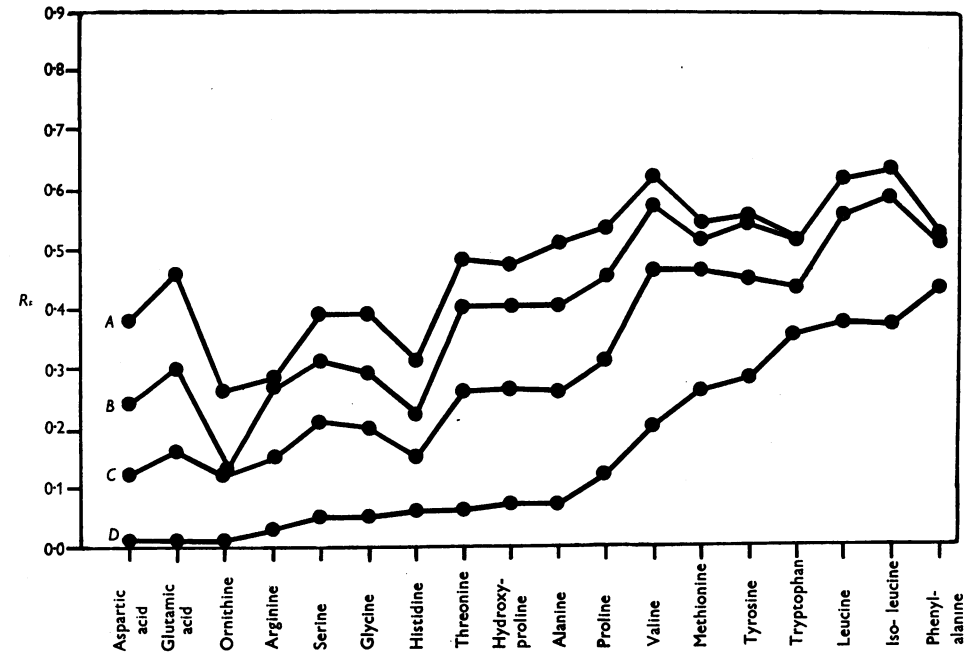


Fig. 2. Comparison of the $R_F$ values of amino-acids in aqueous lower alcohols on Whatman no. 4 paper at room temperature. (*A*, 70% (v/v) methanol; *B*, 70% (v/v) ethanol; *C*, 70% (v/v) *n*-propanol; *D*, *n*-butanol saturated with water.)

position and character of the hydrophilic groups which it contains and also on the water content and the number, position and character of the hydrophilic groups of the solvent.

On this hypothesis it would be expected that, for a given solvent, an increase in water content would result in the solute molecules moving more quickly down the paper, and that the rates of molecules of different solutes would tend to become more nearly equal. The solute molecules would, because of the mass-action effect, have a decreasing affinity for incorporation in the cellulose-water complex as the number of water molecules in the solvent increased and thus have a greater tendency to remain in the mobile phase and move with it. This, in fact, is found to occur (Fig. 1). It is significant that even at very high water contents (80–90 %) the amino-acids, although moving at almost identical rates, do not lie along the solvent boundary, i.e. their $R_F$ values are very nearly, but not quite, equal to unity. It would appear that at these levels there is still some incorporation of solute molecules in the cellulose-water complex.

In the case of the three water-miscible alcohols which we have studied, the increase in hydrophilic character in the series *n*-propanol-ethanol-methanol would be expected to result in progressively greater competition by the alcohol molecules for incorporation in the cellulose-water complex to the exclusion of solute molecules which consequently would have a greater tendency to move with the mobile phase. This effect is apparent from the general increase of $R_F$ values of the amino-acids through this series of alcohols (Fig. 2).

If a mechanism of this type is assumed to be generally operative in paper chromatography then, in the case of solvents only partially miscible with water, the composition of the flowing solvent and of the cellulose-water complex are presumably variable only within fixed limits. The effect will then become almost equivalent to true partition between solvent and water, and $R_F$ values will be expected to correspond with those calculated from the partition coefficients, as found by Consden *et al.* (1944) in the case of *n*-butanol.

## SUMMARY

1. The use of water-miscible solvents for the separation of amino-acids by paper chromatography is described.

2. Aqueous mixtures of relatively non-volatile solvents such as furfuryl alcohol and tetrahydrofurfuryl alcohol give small sharp spots by downward flow in the usual way.

3. With more volatile solvents (lower alcohols, acetone, pyridine and tetrahydrofuran) satisfactory results can be obtained by the method of capillary ascent in small closed glass tanks.

4. Aqueous acetone can with advantage replace collidine for use in conjunction with phenol on two-way chromatograms.

5. The bearing of these results on a possible mechanism for paper chromatography is discussed.

The authors' thanks are due to Miss H. M. Bigg for technical assistance.

## REFERENCES

Arden, T. V., Burstall, F. H., Davies, G. R., Lewis, J. A. & Linstead, R. P. (1948). *Nature, Lond.*, **162**, 691.

Bentley, H. R., Macdermott, E. E., Pace, J., Whitehead, J. K. & Moran, T. (1949). *Nature, Lond.*, **164**, 438.

Bentley, H. R. & Whitehead, J. K. (1949). *Nature, Lond.*, **164**, 182.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Edman, P. (1946). *Ark. Kemi Min. Geol.* **22** A, no. 3.

Goodall, R. R. & Levi, A. A. (1947). *Analyst*, **72**, 277.

Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.

Holtzmann, F. (1936). *Zbl. GewHyg.* **23**, 8.

Karnovsky, M. L. & Johnson, M. J. (1949). *Anal. Chem.* **21**, 1125.

Ludwig, H. (1935). *Arch. Gewerbepath. Gewerbehyg.* **5**, 654.

Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 53.

Williams, R. J. & Kirby, H. (1948). *Science*, **107**, 481.

Wilson, W. C. (1947). *Organic Syntheses*, Collective, **1**, 279. New York: Wiley.