SEPARATION AND ESTIMATION OF THE 2,4-DINITROPHENYL DERIVATIVES OF GLUTAMIC AND ASPARTIC ACIDS

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INTRODUCTION

The separation of the 2,4-dinitrophenyl (DNP) derivatives of the naturally occurring amino acids has been reviewed by FRAENKEL-CONRAT, HARRIS AND LEVY[1], BISERTE, HOLLEMAN, HOLLEMAN-DEHOVE AND SAUTIÈRE[2] and by MONIER[3]. In the course of the studies of the incorporation of the radioactive amino acids into proteins a simple method was required for the quantitative separation of the DNP-glutamic and DNP-aspartic acids at the 0.5 to 5 μmole level. Column chromatographic methods of various kinds have been used for the separation of DNP-amino acids. SANGER[4] introduced chromatography on silica gel. BLACKBURN[5] used buffered columns of the same type. MILLS[6] has used specially treated Celite with chloroform–methyl ethyl ketone mixtures. PERRONE[7] introduced ether and chloroform–butanol solvents with buffered columns of Celite 545. BELL *et al.*[8] mention the use of buffered columns of Hyflo Super Cel with ethyl acetate as solvent. MATHESON[9–11] has shown ethyl acetate to be an extremely useful solvent for the chromatography of the DNP-amino acids on buffered columns and had when this work was begun obtained separations of most of the commonly occurring amino acids with the exceptions of DNP-aspartic and DNP-glutamic acids and DNP-glycine and DNP-proline. More recently he has extended his methods to include the separation of these amino acids[10]. WOOLLEY[12] lists some results of the counter current distribution of some DNP-amino acids between ethyl acetate and acetate buffers and ethyl acetate and phosphate buffer. KHOKHLOV AND CH'IH[13,14] have used butyl acetate–phosphate buffer with counter current distribution to separate and estimate several amino acids.

Of the methods described only the method of PERRONE[7] seemed rapid enough for routine use in isolating DNP-glutamic and DNP-aspartic acids. Some difficulty was experienced in packing the columns using ether as a solvent and complete separation of DNP-aspartic acid, DNP-glutamic acid and DNP-serine on a single column was difficult. CALLOW AND WORK[15] experienced similar difficulties in separation with PERRONE's system and used modified systems for each of the amino acids in which they were interested.

The method to be described is essentially similar to those described by MATHESON[9–11]. The method gives a clear separation between DNP-glutamic and DNP-aspartic acids and removes the derivatives of the other commonly occurring amino acids in a single buffered column of Hyflo Super Cel with a mobile phase of ethyl

acetate–isoamyl alcohol. The method is readily adapted to the estimation of the free amino acids in protein hydrolysates and conditions of quantitative dinitrophenylation are described.

DNP-glutamic and DNP-aspartic acids generally run on paper with very similar R_F values in a variety of solvents[2,3] but can be resolved using phosphate buffer at pH 6 (LEVY[16]) or isoamyl alcohol with acetic acid (BISERTE AND OSTEUX[17]). Conditions are described for the use on paper of the solvent system devised for column chromatography as a useful supplement to the methods of LEVY and BISERTE AND OSTEUX for identification when DNP-hydroxyproline or DNP-*meso*-diaminopimelic acid are present.

METHODS

Column chromatography

Hyflo Super Cel (Johns-Manville and Company) was passed through a sieve of 100 mesh/in., treated with 3 *N* hydrochloric acid for several hours and washed successively with water and ethanol before drying for 24 h at 120–140°.

The solvent mixture was ethyl acetate–isoamyl alcohol–buffer (10:2:5, by vol.). The buffer was 0.1 *M* sodium acetate of pH 4.8 to which was added 3 % (w/v) of sodium chloride. All operations were carried out in a room thermostatically controlled at 20° ± 1° in subdued lighting. 4.0 g of the treated Hyflo Super Cel was suspended in 50 ml of the equilibrated organic phase and shaken for 5 min before the gradual addition of 2.5 ml of the aqueous phase and a further period of shaking for 10 min. The columns of 1 cm internal diameter were packed using a stainless steel rod with a perforated disc[18]. The mixture of DNP-amino acid (10–20 μmoles total, up to 3 μmoles each of DNP-glutamic and DNP-aspartic acids) was applied to the column in 0.6 ml of organic phase. The flow of eluant was 0.4/0.5 ml/min.

Dinitrophenyl derivatives of alanine, cysteine, cystine, glycine, leucine, isoleucine, lysine, hydroxylysine, ornithine, phenylalanine, tryptophan and tyrosine were rapidly eluted followed by those of threonine, serine and hydroxyproline with R values of 1.0, 0.9 and 0.7 respectively. Chromatography of DNP-derivatives of *meso*- and LL-diaminopimelic acid gave R values of 0.7 and 1.2 respectively. DNP-glutamic and DNP-aspartic acids were last to be eluted with R values of 0.50 and 0.28 respectively.

The bands of DNP-glutamic and DNP-aspartic acids were completely separated and could be collected as single fractions. The solvent was removed under reduced pressure at 40° and the DNP-glutamic and DNP-aspartic acids dissolved in 0.01 *M* sodium phosphate buffer of pH 11 for a spectrophotometric estimation at 360 mμ using the molecular extinction coefficients given by LEVY[16].

Preparation of the DNP-derivatives from a mixture of the free amino acids

Reaction of the dicarboxylic amino acids with 1-fluoro-2,4-dinitrobenzene (FDNB) is slow in the presence of other amino acids (*cf.* MILLS[9]) but the following method gave satisfactory recoveries. To the mixture of amino acids (about 15 μmoles total) in 1.0 ml of water were added successively 1.5 ml of ethanol containing 150 μmoles of FDNB and 0.2 ml of 10 % (w/v) sodium bicarbonate; the solution being gently shaken on a water bath at 40° for 6 h. After 4 h a further addition of 0.1 ml of 10 % (w/v) sodium bicarbonate was made. The ethanol was removed under reduced pressure and the excess FDNB and some of the 2,4-dinitrophenol formed were extracted in ether

(3 × 4 ml) and rejected. The DNP-amino acids were extracted in ether (4 × 3 ml) after acidification with 0.5 ml of 5 *N* hydrochloric acid. The ether was removed by evaporation before chromatography. If an excessive amount of 2,4-dinitrophenol had been formed it was readily removed by adding a little water to the sample in a 50 ml beaker and leaving in a freeze-drying apparatus for 24 h.

Paper chromatography

Sheets of Whatman No. 1 paper (36 × 11 cm) were wetted with the buffer solution by descending chromatography and dried at room temperature (20°). The samples were applied in acetone and the papers equilibrated overnight with the aqueous phase before descending chromatography in the organic phase. The R_F values were somewhat dependent on the degree of dryness of the paper, so reference compounds were run. A chromatogram is shown in Fig. 1.

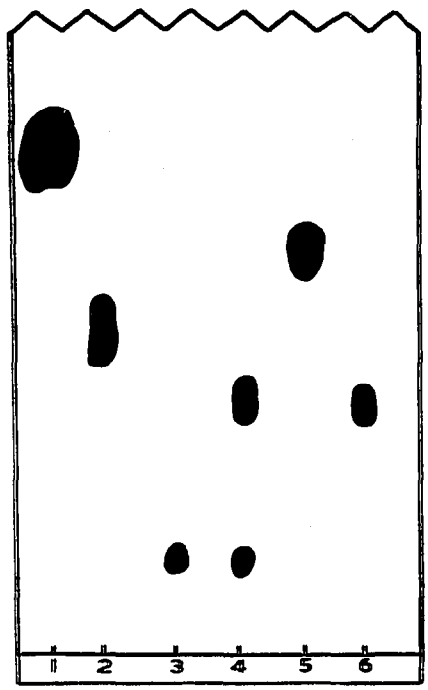

Fig. 1. Chromatogram of DNP-amino acids on Whatman No. 1 paper treated with 0.1 *M* sodium acetate, pH 4.8 and dried. Mobile phase: ethyl acetate–isoamyl alcohol equilibrated with sodium acetate buffer (10:2:5). (1) DNP-hydroxyproline; (2) DNP-*meso*-diaminopimelic acid; (3) DNP-aspartic acid; (4) DNP-glutamic and DNP-aspartic acids; (5) DNP-serine; (6) DNP-glutamic acid.

RESULTS

Efficiency of separation and recovery

The elution pattern of the DNP-glutamic and DNP-aspartic acids from the buffered columns was determined by collecting 0.8 ml fractions of the eluate. The results are shown in Fig. 2. A series of four experiments were carried out in which known amounts of glutamic and aspartic acids were treated with FDNB and their derivatives chromatographed on the buffered columns with spectrophotometric estimation of the eluate. Recoveries were 100 ± 1.0 S.E.M. and 97 ± 0.7 S.E.M. for aspartic and glutamic acids respectively.

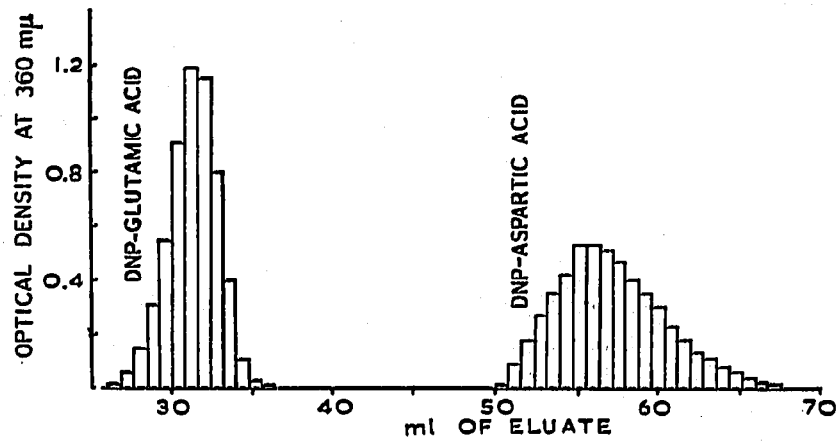

Fig. 2. Plot of absorption at 360 mμ *versus* volume of effluent for a chromatogram of DNP-aspartic and DNP-glutamic acids.

The effect of changes of the pH of the buffer

A series of 0.1 *M* sodium acetate buffers of range 4.4–5.4 were used with the columns to find the optimum pH for the standard method (Table I). Effective separation was obtained at all pH values used with an optimum combination of rapidity of elution with degree of separation at pH 4.8 (before equilibration). The change in R value over 1 pH unit as theoretically expected was close to 10 times.

As acetic acid is appreciably soluble in the organic phase the pH of the aqueous phase is considerably altered after equilibration. The change in pH of the aqueous dhase is given by:

$$\mathrm{pH} = \log (1 + \alpha V/V^l)$$

where V is the volume of organic phase, V^l the volume of the aqueous phase and α the partition coefficient of the acetic acid in favour of the organic phase. The buffer acts effectively if its pK were raised 0.5 units (Table I). Citrate, phosphate and phthalate buffers were used in the same effective range as the acetate. With phosphate and phthalate buffers of the same molarity there was evidence of tailing probably due

TABLE I

RELATIONSHIP OF THE R VALUES OF DNP-GLUTAMIC AND DNP-ASPARTIC ACIDS WITH THE pH OF THE STATIONARY PHASE

Column: Hyflo Super Cel; solvent system: ethyl acetate–isoamyl alcohol–0.1 *M* sodium acetate (10:2:5, by vol.).

Original pH of buffer	*pH of buffer after addition of 3% (w/v) sodium chloride*	*pH of stationary phase after equilibration*	*R values*	
			DNP-glutamic acid	*DNP-aspartic acid*
4.4	4.24	4.98	0.75	0.50
4.6	4.44	5.18	0.63	0.39
4.8	4.65	5.39	0.51	0.28
5.0	4.83	5.57	0.25	0.17
5.2	5.04	5.78	0.12	0.07
5.4	5.24	5.98	0.08	0.04

to inadequate buffering capacity as it could be eliminated by increasing the concentration of the buffers. 1.5 *M* phosphate buffers at pH 6 gave very good separations with narrow bands. At the lower range of pH studied (5.4 after equilibration) the resolution and band sharpness with acetate buffer was better than with the other buffers. Acetate buffer was therefore chosen for the routine method as being slightly more flexible.

The addition of isoamyl alcohol gave a slight increase in the separation achieved. The presence of sodium chloride in the buffer facilitated the separation of the two phases after mixing and appeared to give slightly narrower bands on chromatography. Its use in the method is not strictly essential. A sharpening of the bands was observed on increasing the concentration of the acetate buffer to 1 *M*; the standard deviation of the distribution of the solute in the effluent decreased from 1.6 ml to 1.3 ml for DNP-glutamic and from 2.9 ml to 2.3 ml for DNP-aspartic acid without appreciable effect on the *R* values. Increasing the buffer to 2 *M* gave no further improvement in the distribution of DNP-glutamic acid and spread the DNP-aspartic acid to a S.D. of 3.3.

To avoid the difficulty of removing large quantities of acetic acid in the effluent before the spectrophotometric assay in 0.01 *M* sodium phosphate we used 0.1 *M* sodium acetate to buffer the columns in the standard method as the resolution was adequate.

DISCUSSION

The methods described are simple and inexpensive to carry out and should be useful in problems concerning the dicarboxylic amino acids. In our hands the column chromatography was more convenient and gave clearer separations than the method of PERKOSKE[7].

In contrast to the observations of MATHESON[9,10] in the region of pH 8 the change of *R* value with pH in the region of pH 5 is close to that expected from simple partition of the non-ionised form of the DNP-amino acids.

The superiority of acetate over other buffers at the lower range of pH studied when the *R* values and the concentration of DNP-amino acids in the mobile phase are greater may indicate that there is some association of the DNP-amino acids in the mobile phase which is repressed by the free acetic acid.

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SUMMARY

DNP-aspartic and DNP-glutamic acids can be separated in the presence of other DNP-amino acids on columns of Hyflo Super Cel with 0.1 *M* sodium acetate (pH 5.4

after equilibration) using ethyl acetate–isoamyl alcohol. Some of the factors influencing the separation have been examined. The solvent system may be adapted to paper chromatography.

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